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Kaempferol inhibits *Entamoeba histolytica* growth by altering cytoskeletal functions

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ABSTRACT

The flavonoid kaempferol obtained from *Helianthemum glomeratum*, an endemic Mexican medicinal herb used to treat gastrointestinal disorders, has been shown to inhibit growth of *Entamoeba histolytica* trophozoites in vitro; however, the mechanisms associated with this activity have not been documented. Several works reported that kaempferol affects cytoskeleton in mammalian cells. In order to gain insights into the action mechanisms involved in the anti-amoebic effect of kaempferol, here we evaluated the effect of this compound on the pathogenic events driven by the cytoskeleton during *E. histolytica* infection. We also carried out a two dimensional gel-based proteomic analysis to evidence modulated proteins that could explain the phenotypical changes observed in trophozoites. Our results showed that kaempferol produces a dose-dependent effect on trophozoites growth and viability with optimal concentration being 27.7 μM.

Kaempferol also decreased adhesion, it increased migration and phagocytic activity, but it did not affect erythrocyte binding nor cytolytic capacity of *E. histolytica*. Congruently, proteomic analysis revealed that the cytoskeleton proteins actin, myosin II heavy chain and cortexillin II were up-regulated in response to kaempferol treatment. In conclusion, kaempferol anti-amoebic effects were associated with deregulation of proteins related with cytoskeleton, which altered invasion mechanisms.

1. Introduction

Amoebiasis caused by the protozoan parasite *Entamoeba histolytica* is an important health problem in Mexico and other developing countries in Latin America, Asia and Africa [1]. Taking into consideration the important side effects of the current treatment (Metronidazole) and the decrease in *E. histolytica* drug susceptibility, there is a renewed interest for the use of medicinal plants as a source of therapeutic alternatives. Flavonoids are secondary metabolites of plants with several biological activities. One of them is kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one), a compound with a low molecular weight (MW: 286.2 g/mol) and the classical flavonoid diphenylpropane structure (C6–C3–C6). This molecule has been identified in many vegetal species commonly used in traditional medicine; therefore, it has been the subject of numerous studies. Kaempferol possesses a wide range of biological activities, including antioxidant, anti-inflammatory, anticancer, antibacterial and antiviral activities. Importantly, it is also effective against several protozoa (for review see Ref. [2]). Calzada et al. reported that kaempferol obtained from *Helianthemum glomeratum*, an endemic medicinal herb used to treat diarrhea, abdominal pain and dysentery in Mexico, was one the most active flavonoids against *E. histolytica* and *Giardia lamblia* among 18 plant flavonoids [3]. Later, the same group confirmed that kaempferol was the most active flavonoid against these parasites; its activity was markedly decreased with the presence of a glucosyl moiety [4]. Kaempferol (IC50 = 10.3 ± 2.3 μg/ml) isolated from *Morinda morindoides* leaf extracts, showed a more pronounced antiamoebic activity than its corresponding glycosides, without any cytotoxic effect on MT-4 mammal cells [5]. The effects of kaempferol isolated from *Chresta scapigera*, *Acridocarpus chloropterus* and *Schima wallichii*, as well as chemically synthesized (Fluka, Buchs, Switzerland) against other protozoan parasites,

Abbreviation: IC50, 50% inhibitory concentration.

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including *Leishmania*, *Trypanosoma* and *Plasmodium*, have also been documented [6–9]. Structure-activity analyses suggested that 3-OH and 3′-OH groups on flavonoid contribute substantially to the strong anti-protozoal activity, while the presence of the phenolic group at the C-3 in kaempferol increased cytotoxicity [8]. However, the molecular mechanism underlying the effects of kaempferol on these protozoa remains unknown.

Several studies showed that kaempferol affects cytoskeleton in mammalian cells. Kaempferol enhanced tight junction (TJ) integrity through the cytoskeletal association and expression of TJ proteins in Caco-2 cells [10]. Additionally, kaempferol produced morphological changes in endothelial cells with the formation of cell extensions and filopodias at non cytotoxic concentrations [11]. To our best knowledge, there are not reports about the kaempferol effects on *E. histolytica* cytoskeleton. Pathogenesis of *E. histolytica* has been related to the organization and regulation of the cytoskeleton elements, since trophozoites motility is a key event during invasion of host tissue [12].

With the purpose of identifying molecular events associated with the antiamoebic effects of flavonoids, our group recently reported that the flavonoid (−)-epicatechin alters *E. histolytica* cytoskeleton proteins and functions, which could affect the pathogenic processes of this human pathogen [13]. In order to gain insights into the action mechanisms involved in the anti-amoebic effect of kaempferol, here we evaluated the effect of this compound on the pathogenic events driven by the cytoskeleton during *E. histolytica* infection. We also carried out a two dimensional gel-based proteomic analysis to evidence changes in global protein expression profile that could explain the phenotypical changes observed in the trophozoites.

2. Materials and methods

2.1. Cultures

E. histolytica trophozoites, HM1-IMSS strain, were axenically grown at 37 °C in TYI-S-33 medium, supplemented with 20% bovine serum [14]. Cells were harvested in the log phase of growth for all experiments. Human epithelial colorectal adenocarcinoma cells (Caco-2, HTB-37 ATCC, USA) were grown in advanced minimum essential medium (MEM, Gibco) supplemented with 5% fetal bovine serum, 200 mM glutamine (Gibco), 0.0125% penicillin and 0.02% streptomycin; cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

2.2. Cell growth and viability assays

Kaempferol (Sigma–Aldrich Co.) 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 (1.5×10^5) were grown in complete TYI-S-33 medium containing increasing kaempferol concentrations ranging from 3.9 μg/ml to 15.9 μg/ml (i.e., 13.8–55.5 μM). At 48 h, cells were harvested and resuspended in phosphate-buffered saline (PBS). Trophozoites number was determined in a Neubauer chamber and cell viability was measured using the trypan blue dye method as described [13]. Cells grown in complete TYI-S-33 medium and 0.05% DMSO was used as controls in all experiments. Data were expressed as the mean ± standard deviation (SD) of two independent experiments performed three times.

2.3. Cytotoxicity assays

Caco-2 cells were cultured into a 96-well microplate (3.0×10^4 cells/well) and grown for 24 h. Then, medium was replaced by fresh medium containing 27.7 μM kaempferol or 0.05% DMSO. At 48 h, supernatants were collected, centrifuged at 500 × *g* for 5 min, and transferred to a microtiter plate (50 μl/well) to determine lactate dehydrogenase (LDH) levels using the CytoTox 96® Non-radioactive Cytotoxicity Assay (Promega) following manufacturer recommendations. Cells in free medium and medium with 9% Triton X-100 were used as negative and positive controls, respectively. Cytotoxicity was determined from absorbance values at a 492 nm wavelength and expressed as: [(cells treated with kaempferol or DMSO-cells in free medium)/(cells of positive control-cells of 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 (3.0×104 cells/well) in the presence of 27.7 μM kaempferol or 0.05% DMSO as described above. At 48 h, cells were incubated with 1 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) at 37 °C for 4 h. The medium was removed and formazan dye crystals were solubilized in 100 μl DMSO for 5 min. Cell viability was determined from absorbance at 570 nm wavelength as described [13]. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.5. Adhesion assays

Adhesion of trophozoites to Caco-2 cells was evaluated as described [13,15]. Briefly, Caco-2 cells monolayers were covered with 1 ml serum-free TYI-S-33 medium and incubated with trophozoites previously grown in 27.7 μM kaempferol for 48 h at 37 °C (one amoeba/four Caco-2 cells) for 1 h at 37 °C. Non-adherent trophozoites were collected by washing with serum-free TYI-S-33 medium and counted using the trypan blue dye method. The number of adherent trophozoites was expressed as the difference between the initial number of trophozoites in each well and the number of non-adherent trophozoites. Trophozoites grown in complete TYI-S-33 medium or 0.05% DMSO were used as controls. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

Erythrocytes binding to trophozoites were evaluated as described by Voigt et al. [16] with some modifications [13]. Trophozoites (2.0×10^5) previously treated with 27.7 μM kaempferol or 0.05% DMSO for 48 h at 37 °C were incubated with human erythrocytes (2.0×10^7) for 5 min on ice. Then, amoebas were fixed with 4% paraformaldehyde, free erythrocytes were removed by centrifugation and cells were stained by the Novikoff method [17,18]. Finally, the cellular suspension was observed 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 from 100 randomly selected trophozoites. Experiments were performed twice by triplicated and results were expressed as mean ± SD.

2.6. Cell migration assays

Migration of *E. histolytica* trophozoites (5.0×10^4) previously treated with 27.7 μM kaempferol for 48 h at 37 °C was evaluated by the Transwell migration assay as described [13,19,20]. Trophozoites grown in complete medium or 0.05% DMSO were used as controls. The number of trophozoites that have migrated into the lower chamber was determined using Trypan blue assay. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.7. Caco-2 cells monolayer destruction assays

The cytolytic capacity of *E. histolytica* trophozoites (5.0×10^4) previously treated with 27.7 μM kaempferol for 48 h at 37 °C was evaluated using the CytoTox 96 Non-radiactive Cytotoxicity Assay (Promega) as described [13]. Trophozoites grown in complete TYI-S-33 medium or 0.05% DMSO were used as controls. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.8. Erythrophagocytosis assays

2.8.1. Microscopic evaluation

Trophozoites previously treated with 27.7 μM kaempferol for 48 h at 37 °C were incubated with human erythrocytes (1:100) for 5 and 10 min at 37 °C and engulfed erythrocytes were stained by the Novikoff method as described [13,17,18]. The cellular suspension was observed through a Nikon Eclipse 80i microscope (20–100× magnification). The number of engulfed erythrocytes per amoeba was counted in 100 randomly selected trophozoites and the phagocytic index (percentage of trophozoites that had engulfed erythrocytes multiplied by the average number of erythrocytes ingested per amoeba) was calculated [21]. Trophozoites grown in complete TYI-S-33 medium or 0.05% DMSO were used as controls. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.8.2. Spectrophotometric hemoglobin assay

Amoebas were grown with 27.7 μM kaempferol for 48 h at 37 °C (2.0×10^5) and incubated with human erythrocytes (2.0×10^7) for 10 min at 37 °C. Cells were obtained by centrifugation and non-ingested erythrocytes were bursted by the addition of 1 ml cold distilled water. After centrifugation, the pellet containing only amoebas with completely engulfed erythrocytes was resuspended in 1 ml concentrated formic acid (Sigma) to determine internalized hemoglobin by a colorimetric method through absorbance value at 400 nm [16]. Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.9. Statistical analysis

All the data were processed using the GraphPad Prism 5.01 software. Statistical analyses were performed using one-way ANOVA and Dunnett's and Bonferroni tests. Statistical significance was set at $p \leq 0.05$.

2.10. Two-dimensional gel electrophoresis

Total soluble proteins were extracted from trophozoites (15×10^6) grown in TYI-S-33 medium with or without 27.7 μM kaempferol for 48 h at 37 °C. Cells were lysed in lysis buffer (100 mM Tris, 1 ml; 100 mM E64, 1 ml; 100 mM PMFS, 15 μl; *Complete* proteases inhibitor cocktail Roche, 30 μl), following by three freeze-thaw cycles. After centrifugation (15,000 × g for 5 min at 4 °C), supernatants were obtained and proteins were purified using the ReadyPrep 2D Clean Up kit (Bio-Rad) according to the manufacturer's protocol. Protein pellets were mixed in 100 μl sample buffer (8 M urea, 4% CHAPS, and 80 mM dithiotreitol DTT). Protein concentration and integrity were determined by the Bradford method and 10% SDS-PAGE, respectively.

For two-dimensional gel electrophoresis, protein extracts (400 μg) were mixed with 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). 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 1300 V/h; finally, a hold step at 5400 V/h was applied. Then, 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, proteins were separated by 12% SDS-PAGE for 7 h at 150 V/400 mA. Finally, gels were stained with Bio-safe colloidal Coomassie Blue (BioRad) and fixed with 30% ethanol-10% acetic acid. Three independent biological replicates were performed to assess the reproducibility of the assay.

2.11. Identification of proteins with differential abundance

Images from 2-D gels were documented in an Image Master Amersham GE Life Sciences scanner. The Melanie 7.0 software was used to visualize, explore and analyze gel images in order to detect differential spots between both culture conditions as described [13]. A protein was defined as differentially abundant in both experimental groups according to a cut-off value of at least a 2-fold change in spot volume between matched sets in triplicates and $p < 0.05$ (Student's t test). 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. Protein identity was defined by searches in 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 was $-10 \times \mathrm{Log}(P)$, where P is the probability that the observed match is a random event. The threshold ion score in the above conditions was 41 for $p < 0.05$.

2.12. Western blot assays

Protein extracts (60 μg) from *E. histolytica* trophozoites grown in TYI-S-33 medium with or without 27.7 μM kaempferol for 48 h at 37 °C were resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. After incubation in 5% nonfat milk in PBS-Tween for 1 h at 37 °C, membranes were incubated with PBS-Tween containing rabbit anti-amoebic myosin II antibody (1:750 dilution) or mouse monoclonal anti-human actin antibody (1:900 dilution, MP-Biomedicals) overnight at 4 °C. Then, goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate and goat anti-mouse IgG (H + L) horseradish peroxidase conjugate antibodies (Invitrogen) (1:2000) were used as secondary antibodies (1 h at 37 °C). Mouse polyclonal antibodies against the *E. histolytica* housekeeping lectin (1:750 dilution) were used as internal control. Proteins were revealed by the 3,3′-diaminobenzidine (Sigma–Aldrich) method and bands were submitted to densitometry analysis. Lectin data were used to normalize myosin II and actin data in each condition. Assays were performed twice from three independent experiments.

3. Results

3.1. Kaempferol reduces E. histolytica viability and growth, without affecting Caco-2 cells

To evaluate the effect of kaempferol on *E. histolytica* trophozoites, parasites were incubated with increasing drug concentrations for 48 h, and cell number and cell viability were determined.

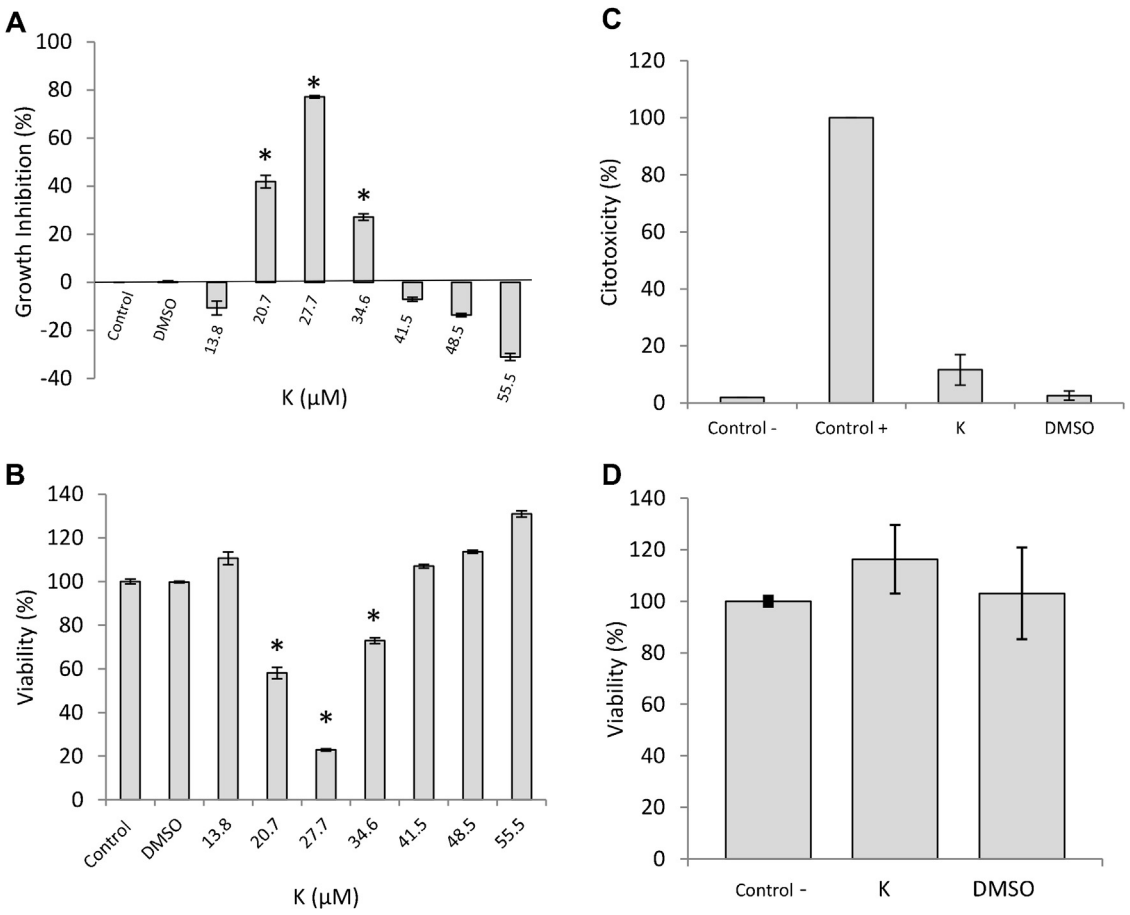

Fig. 1. Kaempferol effect on *E. histolytica* and Caco-2 cells. (A and B) Different concentrations of kaempferol were used to treat *E. histolytica* trophozoites for 48 h at 37 °C. Growth inhibition (A) and cell viability (B) were determined by cellular counting and trypan blue exclusion, respectively. Control cells treated with DMSO and culture medium were included. (C and D) Caco-2 cells were incubated with 27.7 μM of kaempferol for 48 h at 37 °C. Cytotoxicity (C) was evaluated using the Cyto Tox 96 Non-Radiactive Cytotoxicity Kit and cell viability (D) was determined by MTT assay. Cells incubated with MEM (control −), Triton X-100 (control +) and DMSO were included as controls. Experiments were performed twice by triplicate. Data corresponding to mean values ± SD are expressed in percentage in relation to the number of cells grown in culture medium.

Surprisingly, kaempferol seems to have a dual effect on *E. histolytica* since 20.7, 27.7 and 34.6 μM kaempferol produced growth reduction, while lower and higher concentrations promoted cell proliferation. Notably, 27.7 μM kaempferol was the concentration that produced the highest inhibitory effect (77.1 ± 0.6%) on trophozoites growth in comparison with control cells growing with culture medium or DMSO (Fig. 1A).

Congruently, kaempferol showed a dual effect on *E. histolytica* viability according to the trypan blue dye method; 20.7, 27.7 and 34.6 μM kaempferol reduced cell viability, while lower and higher drug concentrations slightly promoted cell viability. Again, 27.7 μM kaempferol was the concentration that produced the highest inhibitory effect (22.9 ± 0.6%) on trophozoites viability in comparison with control cells growing in culture medium or DMSO (Fig. 1B).

To investigate if the most effective anti-amoebic concentration (27.7 μM) of kaempferol has an effect on mammal cells, we evaluated toxicity on Caco-2 cells using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) (Fig. 1C). Results showed that kaempferol produced 11.6 ± 5.4% toxicity on Caco-2 cells, which is not statistically significant in reference to control cells growing with Caco-2 culture medium or DMSO. Additionally, MTT assay showed that kaempferol did not significantly modify Caco-2 cells viability (Fig. 1D).

Taking altogether, these data indicated that 27.7 μM kaempferol produced a considerable reduction of *E. histolytica* growth and viability, affecting slightly Caco-2 cells.

3.2. E. histolytica *adhesion and migrations are affected by kaempferol*

Previous studies have shown that kaempferol affects cytoskeleton in mammalian cells [10,11]. Therefore, we carried out two functional tests to evaluate if kaempferol disturbs cytoskeleton functions in *E. histolytica*. Trophozoites adhesion was evaluated on Caco-2 cells monolayers and by an erythrocyte binging assay. Results showed that only 66.9 ± 5.9% of trophozoites previously treated with 27.7 μM kaempferol were able to adhere to the monolayer of Caco-2 intestinal cells. In contrast, 83 ± 2.2% and 86.2 ± 3.7% of trophozoites grown in medium or 0.05% DMSO, respectively, were found adhered to the target cells (Fig. 2A and B). However, erythrocyte binding affinity was not affected by kaempferol since 51 ± 5.1% of drug-treated trophozoites were able to bind at least one erythrocyte, which is not significantly different from data of the control groups (Fig. 2C and D).

Afterwards, we evaluated the effect of kaempferol on trophozoites migration using the Transwell chamber system. Results

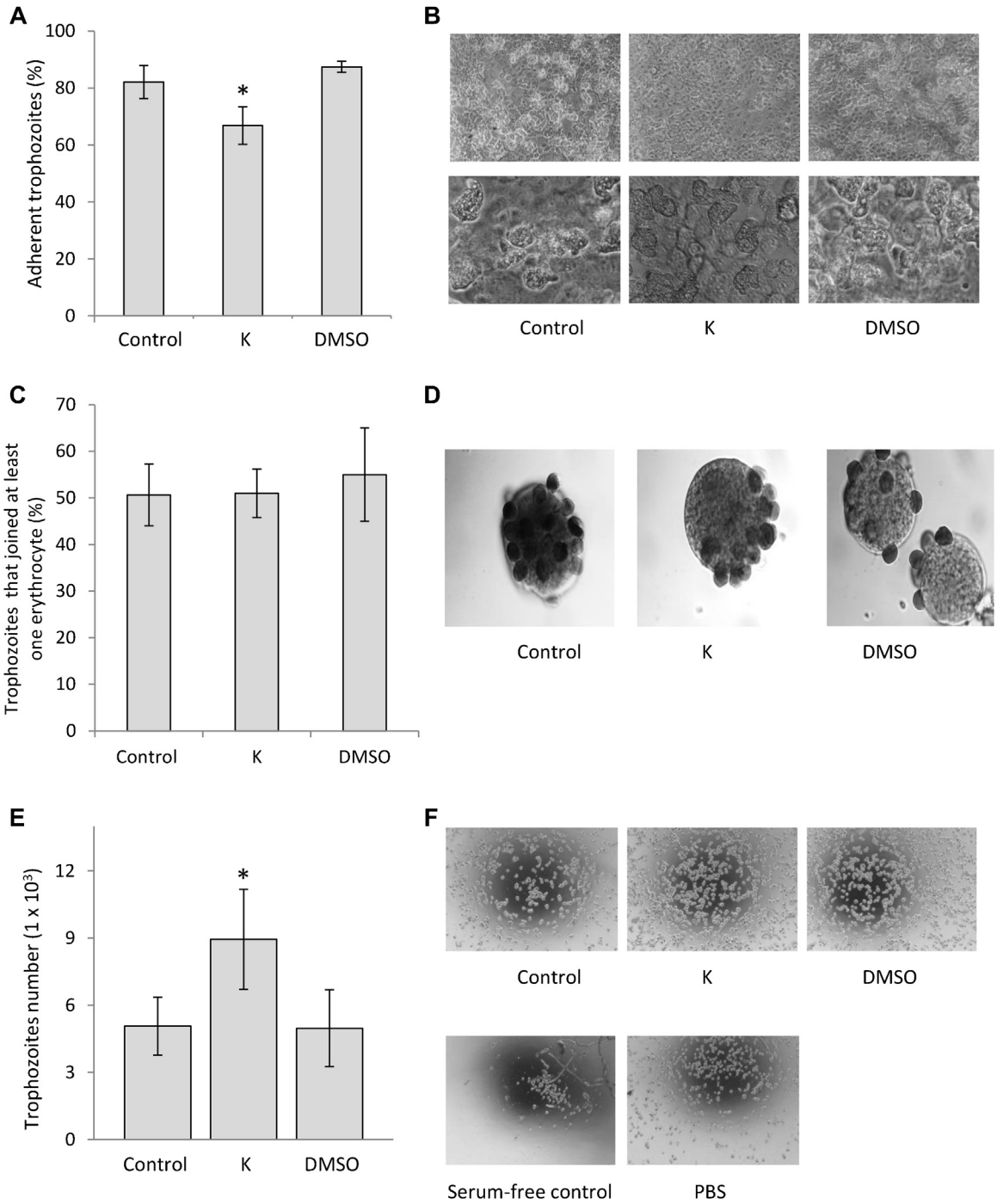

Fig. 2. Effect of kaempferol on *E. histolytica* adhesion, erythrocyte binding and migration. (A) Adhesion assays. Trophozoites previously treated with kaempferol were incubated over Caco-2 monolayers for 1 h at 37 °C and percentage of adherent trophozoites was calculated. (B) Representative micrographs of *E. histolytica* adhered to Caco-2 in each condition (top panel); the bottom panel shows a close-up image of the top panel. (C and D) Erythrocyte binding. Kaempferol-treated trophozoites were incubated with human erythrocytes for 5 min on ice. Joined erythrocytes were evidenced by Novikoff stain (C) and microscopic observation (D). (E) *E. histolytica* migration was determined by the Transwell migration assay and trypan blue staining. (F) Representative images of trophozoites that have migrated into the lower chamber, in each condition. In all experiments, trophozoites without treatment (control) and treated with DMSO were included. Each experiment was performed twice by triplicate. Data shown in A, C and E, correspond to mean values ± SD.

showed that 5068 ± 1291 and 4974 ± 1711 trophozoites incubated in complete medium or 0.05% DMSO, respectively, were able to migrate to the lower chamber. Numbers reached up to 8946 ± 2232 when trophozoites were incubated with 27.7 μM kaempferol (Fig. 2E and F). As controls, only 73 ± 87 and 2544 ± 1261 trophozoites growing in PBS or medium without serum, respectively, were able to migrate (data not shown).

In summary, our results indicated that kaempferol negatively affects adhesion to Caco-2 cells in trophozoites, but conversely migration was increased.

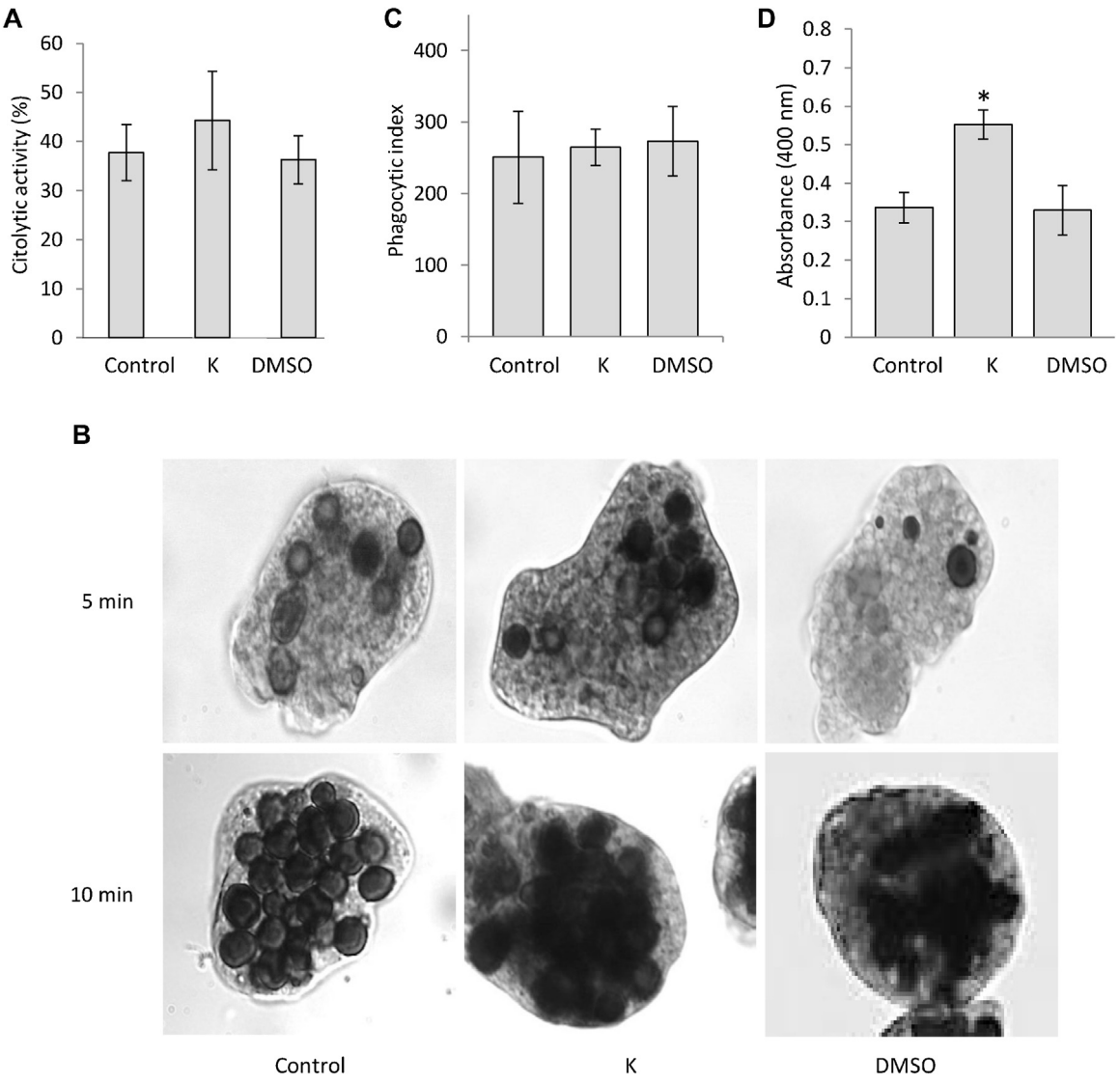

Fig. 3. Effect of kaempferol on *E. histolytica* cytolytic activity and phagocytic index. (A) Cytolytic activity. Trophozoites previously treated with kaempferol were incubated with Caco-2 cell monolayers for 5 min at 37 °C and cytolysis was quantified using the Cito Tox 96 Assay. (B and C) Phagocytic capacity. Trophozoites previously treated with kaempferol were incubated with human erythrocytes for 5 and 10 min and submitted to microscopic evaluation (B). Engulfed erythrocytes were stained by the Novikoff method and the phagocytic index was calculated in each condition (C). (D) Spectrophotometric quantification of hemoglobin. Trophozoites incubated for 10 min with human erythrocytes were bursted and internalized hemoglobin was determined by a colorimetric method through absorbance value at 400 nm. In all experiments, trophozoites without treatment (control) and treated with DMSO were included. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

3.3. *Kaempferol affects erythrophagocytosis but not the cytolytic capacity of* E. histolytica

Other functions related to trophozoite cytoskeleton are cytolysis and phagocytosis. Therefore, we evaluated the effect of kaempferol on the cytolytic capacity of trophozoites on Caco-2 cells using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) (Fig. 3A). We observed that kaempferol treatment (44.26% ± 10.29%) did not significantly affect cytolysis in comparison with control cells growing in medium (37.70% ± 5.8%) or DMSO (36.25% ± 4.9%).

Later, we evaluated the effects of kaempferol on erythrophagocytosis through microscopic evaluation. After 5 min interaction, the phagocytic index corresponding to kaempferol treated trophozoites incubated with erythrocytes was not significantly modified in comparison with control groups. However, when the interaction was maintained for 10 min, the amount of engulfed erythrocytes increased considerably in all groups and it was not possible to determine the exact number (Fig. 3B and D). To overcome this problem, we performed a spectrophotometric assay to quantify internalized hemoglobin in trophozoites. Interestingly, results evidenced that absorbance values at 400 nm increased up to 0.6 ± 0.03 in trophozoites grown in the presence of 27.7 μM kaempferol, whereas they were only 0.3 ± 0.04 and 0.3 ± 0.06 in control cells growing in medium or DMSO, respectively (Fig. 3C). The increased amount of hemoglobin in *Entamoeba* trophozoites indicates the presence of a higher number of engulfed human erythrocytes in parasites treated with kaempferol.

In summary, kaempferol did not affect the cytolytic capacity of trophozoites, while phagocytosis was increased.

3.4. *Kaempferol up-regulates the expression of cytoskeleton proteins in* E. histolytica

Data described above indicated that kaempferol affects trophozoites growth and cytoskeleton functions related to pathogenic

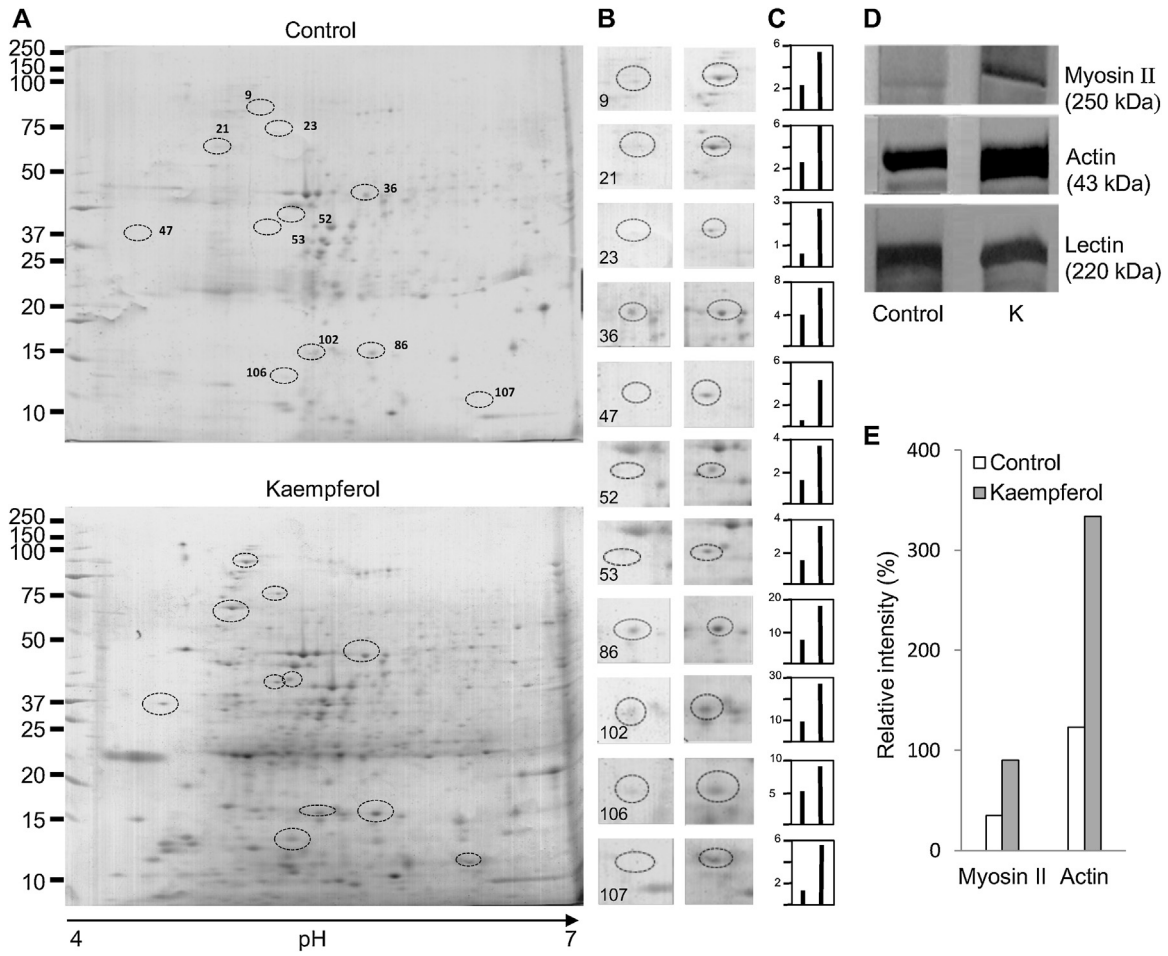

Fig. 4. Proteomic profiling of *E. histolytica* in response to kaempferol. (A) Representative two-dimensional gels of proteins from trophozoite treated (down) or untreated with kaempferol (up). Selected differential spots between both conditions are marked with a circle. (B) Comparative images of selected spots in (A). Left, control cells; right, cells treated with kaempferol. (C) Densitometric analysis of selected spots in (A). Left control spot; right, kaempferol spot. (D) Western blot assay. Immunodetection of myosin II and actin using specific antibodies. (E) Densitometry analysis of bands in (D). Pixels corresponding to the housekeeping lectin protein were considered as 100% in each condition and used to normalize myosin II and actin data.

events. With the purpose of elucidating the molecular mechanisms related to these effects, we searched for differentially expressed proteins in drug-treated parasites through a two-dimensional differential in gel electrophoresis. Since proteomic profiles corresponding to both control groups (trophozoites growing in TY-S-33 medium with or without 0.05% DMSO) were similar [22], trophozoites growing in standard medium were used as control for these assays.

Fig. 4A shows two representative gels from untreated (up) and kaempferol-treated (down) trophozoites. By comparative analysis of 2-D gels, we detected 97 spots with a differential abundance between both conditions. Of these, 59 proteins were up-regulated in the presence of 27.7 μM kaempferol, while 38 proteins were down-regulated. Fig. 4B and C shows the magnification of 11 selected spots whose abundance increased in the presence of kaempferol, with the corresponding densitometry analyses. These spots were excised from Coomassie Blue-stained gels and trypsin digested. Peptides were analyzed through LC/ESI–MS/MS tandem mass spectrometry and protein identity was confirmed by NCBI database searches. Protein name, accession number, fold change, mascot score, matched peptides, molecular mass, sequence coverage, and MS/MS peptide sequence are summarized in Table 1. Interestingly, we identified three proteins associated to cytoskeleton: actin, myosin II heavy chain, and cortexillin II. We also found three proteins involved in the energetic metabolism: aldehyde–alcohol dehydrogenase 2, fructose-1,6-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase, as well as heat shock protein 70, calreticulin and three hypothetical proteins. To validate changes in protein abundance, we performed western blot assays using rabbit anti-amoebic myosin II and mouse monoclonal anti-actin antibodies (Fig. 4D and E). Results confirmed that myosin II and actin were about 1.3 and 2.6-fold over-expressed in response to 27.7 μM kaempferol treatment.

4. Discussion

Searches for new therapeutic compounds against human amoebiasis revealed that kaempferol, a flavonoid that affects cytoskeleton in mammalian cells, has antiamoebic effects [5,23]. However the biological and molecular mechanisms associated with this activity are not described. Here, we obtain a 77.1% ± 0.6% *E. histolytica* growth inhibition using 27.7 μM (7.9 μg/ml) of kaempferol.

The same concentration was previously reported by Calzada [23] as the IC50. Interestingly, the effect was reverted at higher drug concentrations (41.5–55.5 μM) and the number of viable trophozoites significantly increased up to 131.04 ± 1.5%. The same growth promoting effect was also observed at the low concentration of 13.8 μM. A concentration-dependent differential effect has been

Table 1
Up-regulated proteins in response to kaempferol 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
Myosin II heavy chain (9)	C4LU72	2.3	82	2	246709.8/5.2	1.2	[1366]AALSEEIDQANEK [1532]DNQSAEEELNELR
Calreticulin (21)	C4M296	2.8	1037	6	45223/4.69	25	[55]GLQTSEDNKFYIAAAK [71]LDEEFSNKDKNLIVQYNL [89]KFEQGIDCGGGYIK [136]THVIMNYK [172]RIPNPAYK [301]YDSFAYIGIDVWQVKAGTIYDDILITDDIEEAEKEAK
Cortexillin II (23)	N9V0H2	6.4	65	1	61720/5.04	3	[61]TGIVLINFFEIISK
Aldehyde-alcohol dehydrogenase 2 (36)	Q24803	1.8	60	3	96836/7.27	3	[187]LMNHPGVATILATG [201]GNAMVK [235]QAANDVVMSKSFDNGM
Hypothetical protein (47)	B0EMZ1	7.2	177	3	42446/5.03	5	[233]NVFGIVAT [241]QLTEIVPIED [251]EAYKTL
Hypothetical protein (52)	C4M126	3	160	3	37759.0/5.1	15.3	[97]ILNEIVKPLEALVK [126]SINSHNALVTAAEK [226]AVLDQFVVTHIELSTIIADVSQR
Actin protein (53)	B1N2P0	3	89	1	42042/5.26	5	[290]LYGNIVLSGGTSMYPGINTR
Hypothetical protein (86)	C4LWF5	3.4	180	3	15976.9/5.4	30.3	[44]NFFFDQGTQAVYCK [63]ATATAVTDSIAIK [94]GAGGKPHSVVFGDSSER
Fructose-1,6-bisphosphate aldolase (102)	C4LXD7	2.7	704	5	36216/6.1	25.8	[92]GVPISLNLDHGANFQICK [145]FGVTVEGELGVLSGVEDDVAAAEHVFTDPK [175]DVEAFV [182]KDTGVDALAISIGTSHGAYK [209]IRLDILHEVEK
Heat shock protein 70 (106)	Q27646	2.25	1172	7	71782/5.32	20	[81]SDPAIQNDMK [116]KFTPEEISSMVLTK [130]MKETAESFVGK [144]NAVITCPAYFNDSQRQATK [163]DAGTIAGMNVMRIINEPTAAAIAYGLDKK [197]NVLIFDLGGGTFDVSLLAIDDGVFEVK [224]ATNGDTHLGGEDFDNRLVNHFIAEFK
Glyceraldehyde-phosphate dehydrogenase (107)	C4LVR9	5.5	125	1	32216/7.15	8	[149]VINNEFGIVEGLMTTIHATTATQK

[a] UniproKnowledge database.

documented for other flavonoids. In human cancer cells, low concentrations of quercetin stimulated cell proliferation and increased total antioxidant capacity of the cells, while higher concentrations decreased cell survival and viability, thiol content, total antioxidant capacity and enzymatic activities [24]. Likewise, it has been demonstrated that quercetin and fisetin, another flavonoids, produced protective effects against H_2O_2-induced cytotoxicity, DNA strand breaks, and apoptosis at low concentrations (10–25 μM) in rat cells, whereas they induced cytotoxicity, DNA strand breaks, oligonucleosomal DNA fragmentation, and caspase activation at high concentrations (50–250 μM) [25]. In *E. histolytica* cultures, kaempferol seems to show the dual effect previously reported for other flavonoids in mammal cells. This indicates that the selection of the flavonoid concentration is a key determinant for the effects observed.

Having shown that 27.7 μM kaempferol had a slight but no significant toxic effect on human epithelial colorectal cells, we continued with the evaluation of the kaempferol effects on the pathogenic events associated with cytoskeleton functions.

We found that kaempferol affects *E. histolytica* migration and adhesion to Caco-2 cells monolayer, but not erythrocyte binding. Additionally, kaempferol increased phagocytic activity of trophozoites but not their cytolytic capacity. These data indicated that kaempferol treatment alters the molecular mechanisms related to parasite virulence. They also suggested that kaempferol could affect the organization, function and regulation of the cytoskeleton in *E. histolytica*. Congruently, our proteomic analysis revealed that several cytoskeleton proteins were up-regulated in response to kaempferol treatment, namely actin, myosin II heavy chain and cortexillin II. Kaempferol also affected glycolysis and oxidant injury, as evidenced by the modulation of glycolytic enzymes and the heat shock protein 70.

Actin is an essential protein of *E. histolytica* cytoskeleton that interacts with several actin-binding proteins to carry out cellular processes that are relevant for parasite virulence, including adhesion, secretion of proteases and directed locomotion. Notably, in adhesion plates formed during interaction to extracellular matrix proteins, actin filament bundles were found associated with the cell membrane. Actin-binding proteins, such as vinculin, alpha actinin, and myosins I and II were also identified as components of the plates [26]. Myosin proteins function as molecular motors, being essential for cellular movement. Myosins move on actin filaments

converting chemical energy into mechanical work by ATP hydrolysis, producing contraction of the filaments [12]. Myosin II, together with actin, were also reported as components of ligand–receptor complexes or caps formed in the surface of trophozoites [27]. Myosin II is also essential for *E. histolytica* intercellular motility through intestinal cell monolayers and in liver [28]. Cortexillins I and II are a subfamily of proteins with actin-binding sites of the alpha-actinin/spectrin type, described in the social amoeba *Dictyostelium discoideum*. Cortexillins cross-link actin filaments together into bundles, and are required for the cells to maintain their shape and undergo regular cytokinesis [29]. Deletion of cortexillin I and II in *D. discoideum* substantially inhibits the molecular responses to intracellular and extracellular cAMP, and alters the actin cytoskeleton by reducing stimulation of actin polymerization and myosin assembly [30]. *E. histolytica* cortexillin II, has been found upregulated in early phagosomes from wild type amoeba and from parasites that overproduced myosin IB, suggesting its relation with myosin IB activity during the phagocytic process. Additionally, cortexillin II upregulation was reported during chemotaxis signaling by tumor necrosis factor (TNF) in *E. histolytica* [31,32].

The overexpression of actin and myosin proteins has been related to alterations in cytoskeleton functions in different systems, including *E. histolytica*.

Thus, in human colon adenocarcinoma cell line, the increased levels of β- or γ-actin led to actin cytoskeletal remodeling followed by an increase in migration and invasion capacities. These data suggested that expression of both actin isoforms has an impact on cancer cell motility, with the subtle predominance of γ-actin, and may influence invasiveness of human colon cancer [33]. Overexpression of β-actin in myoblasts had striking effects on motility, increasing cell speed to almost double that of control cells; this increased motility appears to depend on myosin function, rather than an increase in the rate of actin polymerization [34].

In *E. histolytica*, the overexpression of the light meromyosin domain of myosin II rendered a dominant negative mutant characterized by abnormal movement, failure to form the uroid and induced capping in the presence of concanavalin A. These defective trophozoites were unable to achieve proper contact with target cells and did not produce the subsequent cellular damage inflicted by control cells [35]. In reference to cortexillin II, there are no reports about the effect of its overexpression, however as it was mentioned before, this protein is related to actin polymerization and myosin II assembly [30].

Based on these data, we hypothesize that the overexpression of actin, myosin II heavy chain, and cortexillin II in response to kaempferol treatment could be producing cytoskeleton remodeling in *E. histolytica* trophozoites, which resulted in significant changes in migration and phagocytic capacity, as well as adhesion, affecting trophozoite pathogenicity. We have previously reported a similar effect for the flavanol (−)-epicatechin on *E. histolytica* trophozoites [13]; like kaempferol, (−)-epicatechin produced an important reduction of adhesion, migration, and phagocytosis. However, unlike kaempferol, (−)-epicatechin produced reduction of cytolytic capacity. The different effects produced by both flavonoids on *E. histolytica* functions and protein expression could be due to their structural differences. (−)-epicatechin and kaempferol belong to different subgroups formed on the basis of structural features of the C ring. Both flavonoids share a hydroxyl group in position 3 of the ring C, however, (−)-epicatechin does not have a double bond between position 2 and 3, and is lacking a carbonyl group, that is, a keto group, in position 4 [36]. Additionally, proteomic analyses revealed that both flavonoids seem to affect glycolysis and oxidant injury, through the modulation of several glycolytic enzymes and the heat shock protein 70.

In conclusion, the flavonoid kaempferol produces a dose-dependent antiamoebic response, deregulating proteins related with cytoskeleton, among others, which promotes changes in invasion mechanisms like adhesion, migration, and phagocytosis. Works currently in progress will help to determine whether kaempferol can affect protein activity, as well as its efficacy in an experimental model of amoebiasis.

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