Curcumin effect on *Acanthamoeba triangularis* encystation under nutrient starvation

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Background: Curcumin is an active compound derived from turmeric, *Curcuma longa*, and is known for its benefits to human health. Amoebicidal activity of curcumin against *Acanthamoeba triangularis* was recently discovered. However, physiological change of intracellular pathways related to a mechanism of *A. triangularis* encystation in the surviving amoeba has never been reported. This data would provide information on the risk of the cyst transformation upon the targeted compound or drug treatment.

Methods: In this study, we are interested in the surviving *A. triangularis* after curcumin treatment to examine the cysts formation by microscopy and evaluate the transcriptional expression of autophagy-related genes. *A. triangularis* autophagy-related proteins have been partially characterized and shown to play a role in the encystation. Thus, starvation was included as an inducer of autophagy and encystation in this amoeba.

Results: Upon autophagy inhibition by 3-methyladenine, a reduction of cysts formation was demonstrated. Interestingly, most of the parasites remained in the trophozoites stage upon curcumin treatment, even under the starved condition. A percentage of trophozoites with enlarged vacuoles was significantly increased in the starved condition. However, in the presence of curcumin, the percentage decreased significantly. Moreover, real-time PCR revealed that the mRNA expression of *A. triangularis* autophagy-related genes, ATG3, ATG8b, ATG12, ATG16, under the starvation with curcumin was at a basal level. The results were similar to the Curcumin-treated amoeba under a nutrient-rich condition, except the *Ac*ATG16 increased at a later time. Altogether, the data reveal that curcumin stress does not induce cysts formation in the surviving trophozoites, which may result from the low expression of key ATG-related genes. However, further investigation into the mechanism of curcumin in the *A. triangularis* trophozoites arrest and its association with autophagy is needed to support the future use of curcumin.

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Abstract

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Results: Upon autophagy inhibition by 3-methyladenine, a reduction of cysts formation was demonstrated. Interestingly, most of the parasites remained in the trophozoites stage upon curcumin treatment, even under the starved condition. A percentage of trophozoites with enlarged vacuoles was significantly increased in the starved condition. However, in the presence of curcumin, the percentage decreased significantly. Moreover, real-time PCR revealed that the mRNA expression of *A. triangularis* autophagy-related genes, ATG3, ATG8b, ATG12, ATG16, under the starvation with curcumin was at a basal level. The results were similar to the Curcumin-treated amoeba under a nutrient-rich condition, except the *Ac*ATG16 increased at a later time. Altogether, the data reveal that curcumin stress does not induce cysts formation in the surviving trophozoites, which may result from the low expression of key ATG-related genes. However, further investigation into the mechanism of curcumin in the *A. triangularis* trophozoites arrest and its association with autophagy is needed to support the future use of curcumin.

Subjects Parasitology, Cellular and Molecular Biology, Microbiology

Keywords Autophagy, *Acanthamoeba triangularis*, Curcumin, Encystation, Nutrient Starvation, Real-time PCR

Introduction

Acanthamoeba spp. are free-living amoeba present in the environment, particularly soil and water *(Siddiqui & Khan 2012)*. Several species of *Acanthamoeba* have been characterized *(Chelkha et al. 2020)*, and most of the human pathogenic species are classified into T4 genotype, for example, *Acanthamoeba castellanii*, *A. polyphaga*, and *A. triangularis (Guimaraes et al. 2016; Hussain et al. 2020; Juarez et al. 2018)*. *Acanthamoeba* spp. are transmitted to humans by different routes *(de Lacerda & Lira 2021; Neelam & Niederkorn 2017; Rayamajhee et al. 2021)* and lead to various clinical presentations, especially in immunocompromised individuals that may present with granulomatous amebic encephalitis *(Matson et al. 1988)*, chronic sinusitis *(Kim et al. 2000)*, or cutaneous lesions *(Morrison et al. 2016)*. In addition, to the healthy individuals who wear contact lenses, this group is at risk of *Acanthamoeba* infection if they have poor personal hygiene habits and *Acanthamoeba* keratitis (AK), a well-known ocular disease caused by this protozoan parasite, usually present in the group of people *(Khan et al. 2019; Lorenzo-Morales et al. 2015; Neelam & Niederkorn 2017)*. Regarding *Acanthamoeba* life cycle, the amoeba usually presents with trophozoites stage, a metabolically active form and can multiple within the human host. However, after being exposed to a stressful condition, it can transform to cyst form with a double wall that is more resistant to a harsh environment *(Anwar et al. 2018)*. This form is a major barrier to *Acanthamoeba* treatment. Even a number of drugs have been approved by the United States Food and Drug Administration so far, but standard therapeutic management of *Acanthamoeba*-infected patients is not yet available *(Elsheikha et al. 2020)*. The two most common first-line drugs currently used for *Acanthamoeba* treatment, especially in AK patients, still use chlorhexidine and polyhexamethylene biguanide. However, identifying new compounds and screening natural extracts for amoebicidal activity are still attractive approaches for more studies. It could provide an alternative drug for *Acanthamoeba* treatment or be used as a complementary treatment of *Acanthamoeba* infection in the future.

Autophagy is a lysosomal degradation pathway for intracellular cytosolic materials *(Feng et al. 2014; Yorimitsu & Klionsky 2005)*. This mechanism is essential for all eukaryotic cells to supply energy and support cell survival. In humans, a defect of the autophagy process is associated with several diseases, for example, neurodegenerative diseases *(Menzies et al. 2015)*, non-alcoholic fatty liver disease *(Khambu et al. 2018)*, or infectious diseases *(Brinck Andersen et al. 2020; Castillo et al. 2012)*. Starvation or nutrient depletion is a classical stress condition for autophagy induction both *in vitro* and *in vivo (Mizushima et al. 2004; Suzuki 2013)*. Several autophagy-related (Atg) proteins participate in the formation of a double-membrane vacuole called autophagosome *(Eskelinen 2005; Feng et al. 2014)*. In mammals, more than 30 Atg proteins have been identified *(Feng et al. 2014)*. However, a partial list of ATG genes are conserved in free-living amoeba, including *Acanthamoeba* spp., and some Atg proteins have been identified *(Kim et al. 2015; Moon et al. 2009; Picazarri et al. 2008; Song et al. 2012)*. *Acanthamoeba* autophagy is of interest as a number of Atg proteins have been partially characterized and reported to be involved with *Acanthamoeba* encystation, a mechanism in which trophozoites transform to cysts *(Kim et al.*

2015; Moon et al. 2011; Moon et al. 2013; Song et al. 2012). Hence, a study of autophagy at both transcriptional and protein levels is needed to understand its biological functions and interaction with other intracellular pathways, which further extends to its association with their pathogenesis in humans.

Curcumin, an active compound obtained from turmeric, *Curcuma longa (Kocaadam & Şanlier 2017)*, contains several pharmacological activities, for example, anti-inflammatory *(Wal et al. 2019)*, anti-oxidant *(Jakubczyk et al. 2020)*, anti-cancer *(Tomeh et al. 2019; Vallianou et al. 2015)*, and antimicrobial activities *(Cui et al. 2007; Martins et al. 2009; Mitsuwan et al. 2020a; Teow et al. 2016; Yang et al. 2016)*. The amoebicidal activity of curcumin against *A. triangularis* trophozoites and cysts have been recently identified *(Mitsuwan et al. 2020a)*. It reveals another property of curcumin against this water-borne parasitic pathogen and could be a promising compound for further drug development against *Acanthamoeba* infection. In this study, we investigated the stress induced by curcumin on the surviving *A. triangularis* trophozoites by microscopy examination of cysts formation and molecular analysis of autophagy-related as well as other encystation-related genes at the transcriptional level. This raises another point of concern in addition to the cidal activity of curcumin and provides insight into the autophagy mechanism in *A. triangularis* in response to curcumin stress to indicate a risk associated with the use of curcumin for *Acanthamoeba* infection.

Materials & Methods

Curcumin preparation and determination of the half-maximal inhibitory concentration (IC_{50})

Curcumin powder was commercially purchased (Sigma Aldrich, St. Louis, USA). The curcumin was dissolved in 100% DMSO and prepared at stock 750 mg/mL. This further diluted with the DMSO as appropriate. Identification of IC_{50} against *A. triangularis* trophozoites was performed in 96-well black plate (SPL Life Sciences, Seoul, Korea). Curcumin concentration was prepared with 2-fold serial dilution with starting final concentration of 8,000 µg/mL. Trophozoites were harvested and washed with fresh AnaeroGRO™ Peptone Yeast Extract Glucose Broth: Proteose peptone and yeast extract were purchased from HiMedia Laboratories, Mumbai, India. Sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), disodium phosphate ($NaHPO_4$), sodium chloride (NaCl), calcium chloride ($CaCl_2$), and glucose were from Sigma Chemical Co. (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4) and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) were procured from Labscan (Bangkok, Thailand). Trypan blue (0.4%) was obtained from Gibco BRL (Grand Island, NY, USA). All chemicals and medium components used were of analytical grade and added at $2x10^4$ cells/well. A control group of untreated cells and PYG medium alone was included. All edge wells were filled with Page's saline (PAS) buffer. After 24 h post-treatment, the parasite viability was analyzed by PrestoBlue® reagent (Invitrogen, Waltham, USA) staining according to the manufacturer's protocol. The plate was incubated for 30 min at 37°C incubators, and fluorescence intensity was measured at excitation/emission wavelength of 535/615 nm by a

153 microplate reader (BioTek SynergyTMMX microplate reader, Winooski, VT, USA). Curcumin
154 IC_{50} was then calculated by prism5 software (GraphPad Software, CA, USA). The experiments
155 were conducted in triplicate with 3 independent experiments.
156
157 ***A. triangularis*** **cultivation**
158 PYG medium, a nutrient-rich condition or full medium, [2% (w/v) proteose peptone, 0.1% (w/v)
159 yeast extract, 400 μM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 50 μM
160 $(NH_4)_2Fe(SO_4)_2$, 100 mM glucose] was used to grow *A. triangularis* trophozoites, strain WU19001
161 *(Mitsuwan et al. 2020a)*. The parasite was maintained at room temperature (RT) in the dark without
162 shaking *(Taravaud et al. 2017)*. The culture medium was replaced with fresh PYG every 2 days
163 until trophozoites harvesting. To induce *A. triangularis* cysts, trophozoites were washed and grown
164 in PAS supplemented with 5% glucose *(Aqeel et al. 2013)*, a nutrient-depleted condition, called
165 starvation. The PAS powder, obtained from HiMedia, Mumbai, India, consisted of NaCl,
166 $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, Na_2HPO_4, KH_2PO_4.
167
168 **Analysis of cysts formation and vacuolization**
169 *A. triangularis* trophozoites were cultured in PYG medium or PAS supplemented with 5% glucose,
170 and the curcumin was added at a final concentration of 50 μg/mL for 24 h. After Trypan Blue
171 staining, the parasites were assessed for cysts formation and vacuolization every 6 h after the
172 curcumin treatment. At least 200 viable cells per condition were investigated, and different forms
173 of the parasites i.e. irregular trophozoites, rounded trophozoites, and cysts, were identified under
174 a light microscope. The percentage of cyst and proportion of parasite forms at each time point was
175 calculated. The irregular trophozoites were further evaluated for their vacuole formation, and the
176 surviving trophozoites of at least 100 cells per condition were analyzed. The trophozoites with
177 vacuoles, regardless of their size, as well as the trophozoites containing enlarged vacuoles, were
178 examined. An enlarged vacuole *(*EV*)* was defined as a vacuole with a diameter of at least 5 μm,
179 and the trophozoite containing at least 1 EV was counted as 1 *(Boonhok et al. 2021b)*. The
180 experiment was performed with 3 independent experiments.
181
182 **Determination of minimal inhibitory concentration (MIC) and drug combination assay**
183 A drug combination study of chlorhexidine, a standard anti-*Acanthamoeba* drug, and curcumin for
184 their amoebicidal activity was performed. The minimum inhibitory concentration (MIC) of
185 curcumin and chlorhexidine was first identified along with the microtiter broth dilution method
186 *(Mitsuwan et al. 2020a)*. The drug/compound was prepared in 96-well clear plate (SPL Life
187 Sciences, Seoul, Korea), and trophozoites of 2×10^5 cells/100 μL were then added into each well.
188 The plates were incubated at RT in the dark for 24 h. The parasite viability was quantified by
189 Trypan Blue staining under a light microscope, Eclipse TE2000-S (Nikon, Tokyo, Japan). The
190 MIC value in our study referred to the lowest concentration with *A. triangularis* growth inhibition
191 greater than 90%. Thus, the MIC of curcumin and chlorhexidine was 250 and 16 μg/mL,
192 respectively. Their MICs were used in drug combination as-say as a starting concentration. The

drug and curcumin were prepared in 96-well plate with 2-fold serial dilution. Then, the trophozoites were added and incubated at RT for 24 h before quantification of parasite viability. To examine the effect of curcumin in combination with autophagy inhibitors, 3-methyladenine (3MA) and wortmannin, which were purchased from Sigma Aldrich, St. Louis, USA, the drug combination assay was then performed as mentioned above. However, the starting concentration of the inhibitors was used at 20 µg/mL to cover the concentration tested in this study.

Preparation of total RNA and cDNA synthesis

A. triangularis trophozoites were cultured with PYG medium or PAS supplemented with 5% glucose medium in a 24-well transparent plate with a final number of $2x10^5$ cells per well. The parasites were treated with curcumin at a final concentration of 50 µg/mL and incubated at RT for 24 h. The parasites were harvested at different time points i.e. 6, 12, 18, and 24 h after treatment. Each time point, the parasites of untreated and curcumin-treated cells were harvested, transferred to 1.5 mL Eppendorf tube separately, and kept on ice. The cell suspension was then centrifuged, and the medium was discarded. The parasite pellets were vortexed, and TRI reagent (Molecular Research Center, Cincinnati, USA) of 500 µL was immediately added to lyse the cell pellet and preserve the parasite's RNA. The total RNA extraction was performed using RNA extraction kit (Vivantis Technologies, Selangor, Malaysia), and a 100 ng mRNA was converted to cDNA by Viva cDNA synthesis kit (Vivantis Technologies, Selangor, Malaysia) following the manufacturer's protocol. Then, the cDNA was kept at -20°C until use.

Validation of PCR primers

All specific primers against *Acanthamoeba* genes used in this study are listed in Table S4. Target genes were ATG3 (GenBank accession no. GU270859), ATG8b (GenBank acces-sion no. KC524507.1), ATG12 (GenBank accession no. HQ830265.1), ATG16 (GenBank ac-cession no. FJ906697), cellulose synthase (CS) (GenBank accession no. EDCBI66TR), serine proteinase (SP) (GenBank accession no. EU365404), metacaspase (MCA) (GenBank acces-sion no. AF480890), interleukin-1 converting enzyme-like protease (IL) (GenBank accession no. XM_004338552), and 18S rRNA was used as a reference gene. These primers were tested against *A. triangularis* strain WU19001 DNA. To confirm primer specificity, the PCR product was sent for sequencing (Apical Scientific Sdn. Bhd., Selangor, Malaysia), and the DNA sequence was then analyzed and blasted against *A. castellanii* NCBI databases before performing a quantitative PCR *(Boonhok et al. 2021a)*.

Analysis of gene expression by quantitative PCR

iTaq Universal SYBR Green Supermix Kit obtained from Bio-Rad (Bio-Rad, Hercules, USA) was used to prepare quantitative PCR (qPCR) reaction along with the manufacturer's instruction. The 18S rDNA was used as a reference gene. The reaction was conducted in PCR tube, which consisted of 10 µL of 2X iTaq Universal SYBR Green Supermix, 100 ng cDNA, and 1 µL of 200 mM F+R primers. The total volume was adjusted with DEPC water up to 20 µL. Thermal cycler,

StepOnePlus Real-time PCR systems (Applied Biosystems, Waltham, USA), software was set as follows; holding stage 95°C for30 s, cycling stage for 40 cycles at 95°C for 15 s, 60°C for 60 s, and melting curve stage at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s with a temperature increase of 0.3ºC. The average deltaCt (ΔCt) was obtained by the thermal cycler. The delta-delta Ct (ΔΔCt) and a relative expression of the mRNA were calculated as follows, the ΔΔCt = [(Ct of treated sample GOI - Ct of treated sample housekeeper) - (Ct of untreated control GOI - Ct of untreated control housekeeper)] where GOI refers to the gene of interest. The relative mRNA expression = 2 to the power of (minus X) or 2^{-X} where X is ΔΔCt. Interpretation of the result is if the value > 1, < 1, and 1 mean the expression is increased, decreased, and constant, respectively.

Statistical data analysis

The experiments were conducted with 2-3 technical replicates in 3 independent experiments. All data were recorded in Microsoft Excel 2016 (Microsoft Corporation, Washington, USA). The statistical analysis was done by Prism 5 software (Graphpad Software, San Diego, USA), and the mean ± SD or ± SEM, including a two-tailed unpaired Student's t-test was used. *P* values of less than 0.05 were considered statistically significant.

Results

Starvation induces *A. triangularis* encystation

Starvation or nutrient-depleted condition was used to induce *A. triangularis* encystation. The mean percentage of *A. triangularis* cysts was approximately 53.30%, and it was significantly different from that of the nutrient-rich condition or full medium (Fig. 1A). The representative images of the parasites under starved and full conditions are shown in Fig. 1B.

Effect of Curcumin on *A. triangularis* autophagy under starvation

IC_{50} of curcumin against *A. triangularis* trophozoites under the full condition at 24 h treatment was first identified, and the IC_{50} was 48.64 ± 30.86 µg/mL. The representative data is shown in Fig. S1. The curcumin concentration of 50 µg/mL was then used as representative curcumin concentration throughout this study. Starvation alone was included as a positive control for *A. triangularis* encystation. Starvation is known as a classical autophagy inducer in several eukaryotic cells *(Díaz-Troya et al. 2008; Kamada et al. 2004)*; thus, in this assay, we included autophagy inhibitors, 3MA, and wortmannin, to examine a physiological and morphological change of *A. triangularis* upon the treatment. Our result showed that in the presence of 1 mM 3MA, the encystation was significantly decreased, whereas the 1 µM wortmannin was slightly impacted to the encystment. The percentage of cysts under starvation + 3MA or wortmannin was approximately 22.55% and 41.30%, respectively (Fig. 2A). We next investigated whether curcumin stress supports cysts formation under starved conditions. Interestingly, in the presence of 50 µg/mL curcumin, the percentage of cysts was approximately 1.39%, the surviving amoeba remained in the trophozoites stage (Fig. 2A). The representative images of the curcumin-treated

parasite under starved conditions are shown in Fig. 2B. In addition, the parasites treated with a combination of curcumin and autophagy inhibitors were included in this experiment to test whether this could completely inhibit the encystation or not. The result showed that the percentage of cysts in curcumin+3MA and curcumin+wortmannin remained at the basal level similar to that of curcumin-treated alone (Fig. 2A). Moreover, different forms of the viable *A. triangularis*, i.e. irregular trophozoites, rounded trophozoites, and cysts under different conditions, starvation alone, starvation + 3MA, and starvation + curcumin, were quantified under the microscope every 6 h. In starvation alone, the irregular trophozoites started to round at 6 h. Cysts were seen at 12 h after culture, and at 24 h, the percentage of cysts was approximately 50%. In the presence of 3MA, cysts were clearly seen at 18 h post-treatment, and at 24 h, the percentage of cysts was approximately 20%. In curcumin-treated conditions, the parasites were mainly in irregular trophozoites, approximately 90%, and the percentage of cysts was approximately 2% (Fig. S2).

Evaluation of vacuolization in surviving trophozoites was further performed. The number of the trophozoites containing vacuole and trophozoites with enlarged vacuole were analyzed under the light microscope. The percentage of trophozoites with vacuoles in curcumin-treated condition was almost 100% along with the 24 h treatment, and the percentage was similar to those of starvation alone and starvation + 3MA (Fig. S3A). The percentage of trophozoites with enlarged vacuoles was further investigated. In starvation alone, the percentage was significantly increased along with the treatment, and the mean percentage at 24 h was approximately 22.41%. Interestingly, in curcumin-treated condition, the mean percentage along the treatment was significantly reduced and maintained in the range of 7.89-12.16%. The result was similar to that of 3MA-treated condition in which the percentage was in the range of 6.79-10.92% (Fig. 2C).

Molecular analysis of *A. triangularis* autophagy-related genes, ATG3, ATG8b, ATG12, and ATG16, at transcriptional level upon curcumin treatment, was conducted. Validation of PCR primers (Table S4) by conventional PCR against *A. triangularis* DNA was first performed, and the target genes were successfully amplified. The gel result representing PCR products is shown in Fig. S4. In addition, analysis of DNA sequencing of the amplicons was performed, and the results are shown in Table S5. Then, the quantitative PCR was performed, and the results showed that the mRNA expression of all tested ATG genes was unchanged along with the treatment and maintained at the basal level (Fig. 2D). In addition, the expression of these ATG genes under 3MA-treated conditions was investigated. As expected, the expression of the ATG genes was at the basal level along with the treatment (Fig. S5). The overall results demonstrated the inhibitory effect of curcumin in the surviving trophozoites against *A. triangularis* encystation even under starvation. The effect is possibly resulting in no upregulation of the ATG genes and reduction of parasites number with enlarged vacuoles.

Effect of Curcumin on *A. triangularis* autophagy under a nutrient-rich condition

To measure the effect of curcumin alone without the stress from starvation, the *A. triangularis* trophozoites were cultured in PYG, a nutrient-rich medium. As expected, curcumin did not activate cysts formation. The percentage of cysts was at a basal level and did not significantly different from that of the full medium alone (Fig. 3A). The representative image of the parasites under curcumin treatment is shown in Fig. 3B. The surviving parasites remained in the trophozoites stage.

Vacuolization in the surviving trophozoites was then analyzed. The percentage of trophozoites with vacuoles under curcumin treatment was nearly 100%, and the percent-age was at a comparable level to full medium alone (Fig. 3C). To investigate the maturation of vacuole, the parasites with enlarged vacuoles were examined. The percentage of trophozoites with enlarged vacuoles was consistent along with the treatment, in the range of 6.23-6.81%.

Molecular analysis by qPCR revealed that mRNA expression of the autophagy-related genes i.e. ATG3, ATG8b, ATG12 genes were at the basal level throughout the treatment while ATG16 mRNA expression was increased at 18 and 24 h post-treatment (Fig. 3D). Altogether, the curcumin maintained the parasites at the trophozoites stage, and the mRNA expression of the *A. triangularis* ATG genes was not up-regulated except ATG16. These demonstrated the effect of curcumin on the *A. triangularis* trophozoites without interference with starvation stress.

Effect of Curcumin on *A. triangularis* encystation-related genes under a nutrient-rich condition

Apart from autophagy, we further assessed other *A. triangularis* encystation-related genes i.e. cellulose synthase (CS) and serine proteinase (SP) upon curcumin treatment. The mRNA expression pattern of both genes was similar in that the expression was slightly changed during the first 18 h, and significantly increased at 24 h post treatment (Fig. 4). Moreover, due to a crosstalk between autophagy and apoptosis in other eukaryotic cells, we further observed the mRNA expression of genes involved with apoptosis pathway i.e. metacaspase (MCA) and interleukin-1 converting enzyme-like protease (IL) in response to curcumin. MCA mRNA expression was slightly changed but still at the basal level along with the treatment. The expression of IL mRNA was rapidly increased at 6 h post-treatment and gradually declined at later time points. However, the increased expression was again observed at 24 h post-treatment (Fig. S6). Altogether, from the microscopic examination to molecular analysis, the response of surviving *A. triangularis* to curcumin either under starvation or a nutrient-rich condition was illustrated in Fig. 5.

Curcumin-based drug combination study

A drug combination study between curcumin and chlorhexidine was performed, and to our previous results on the co-treatment of curcumin and autophagy inhibitors, 3MA and wortmannin in which it did not completely inhibit *A. triangularis* encystation, thus theses combinations were

included in this assay to see their interaction. The concentration of compound/drug was varied based on their MICs except for the autophagy inhibitors that were designed to cover the concentration used in the previous experiment. The MICs of curcumin and chlorhexidine were started at 250 and 16 µg/mL, respectively. These were used as a starting concentration in drug combination assay while the starting concentration of 3MA and wortmannin was used at 20 mM and 20 µM, respectively. The results of parasite viability were represented as mean±SD. In curcumin-chlorhexidine combination assay, at maximum concentrations of curcumin (MIC 250 µg/mL) and chlorhexidine (MIC 16 µg/mL), the percentage viability of trophozoites was in a range of 5-8%. Reduction of chlorhexidine concentration to 8 µg/mL in combination with different concentrations of curcumin, the percentage was increased into the range of 42–53%, but their percentages were similar to that of chlorhexidine alone, at 52%. At lower concentrations of chlorhexidine (4, 2, 1 µg/mL), a pattern of the percentage viability at certain chlorhexidine concentration was similar, and their percentage viability was gradually increased when the curcumin concentration was reduced (Table S1). In curcumin-3MA (Table S2) and curcumin-wortmannin (Table S3) combinations, the result pattern was similar. At certain 3MA or wortmannin concentrations below the curcumin's MIC, the percentage viability was gradually increased when reducing curcumin concentration. To the combinations that were close to our interest, 62.5 µg/mL curcumin-1.25 mM 3MA or 1.25 µM wortmannin, the percentage viability was at a comparable with curcumin alone. Taken together, no synergistic, additive, or antagonistic effects were observed in any drug combinations against *A. triangularis* trophozoites.

Discussion

The cystic stage of *Acanthamoeba* is one of the major obstacles for therapeutic use as the penetration of anti-*Acanthamoeba* drugs across a double-layered cyst wall is fairly difficult *(Abjani et al. 2016; Turner et al. 2000)*. Identification of new active compounds and drug repurposing with the amoebicidal activity are urgently needed. In addition, the compound/drug that is able to prolong the trophozoites stage might be useful for drug combination purposes in *Acanthamoeba* infection. Our study has screened several medicinal plants, including curcumin, for the anti-*Acanthamoeba* activity. In this study, the cidal activity was confirmed, and interestingly, the surviving *A. triangularis* after curcumin treatment was arrested at the trophozoites stage. The dual benefits of curcumin, amoebicidal activity, and arresting cyst transformation against *Acanthamoeba* have got more attention. Regarding a long history of curcumin in medicinal use, it contains several pharmacological activities, for example, anti-inflammatory *(Wal et al. 2019)*, anti-oxidant *(Jakubczyk et al. 2020)*, anti-cancer *(Tomeh et al. 2019; Vallianou et al. 2015)*, and antimicrobial activities *(Cui et al. 2007; Martins et al. 2009; Mitsuwan et al. 2020a; Teow et al. 2016; Yang et al. 2016)*. To its anti-parasitic effect, the curcumin has been very-well studied in many parasites for example *Schistosomiasis mansoni (de Paula Aguiar et al. 2016; Hussein et al. 2017)*, *Besnoitia besnoiti (Cervantes-Valencia et al. 2019)*, *Giardia lamblia (Gutiérrez-Gutiérrez et al. 2017)*, *Leishmania major (Koide et al. 2002)*, *Plasmodium falciparum (Cui et al. 2007; Mishra et al. 2008)*, and *Trypanosoma cruzi (Novaes et al. 2016)*. The killing mechanism by curcumin was partially characterized in some parasites. However, this documentation is largely unknown in *Acanthamoeba*. In this study, the curcumin effect in arresting cysts transformation on surviving *A. triangularis* is our main interest by focusing on *Acanthamoeba* autophagy. The curcumin IC_{50} against *A. triangularis* trophozoites was first identified, and the average concentration was 48.64 ± 30.86 µg/mL. The IC_{50} concentration was at a similar level as tested against another amoeba, *Naegleria fowleri*, which is a brain-eating amoeba. Its IC_{50} was 74 µM *(Mungroo et al. 2020)*. Then, to study the effect of curcumin on the surviving *A. triagularis*, the concentration of 50 µg/mL was applied throughout this study.

Encystation refers to a mechanism in which amoeba trophozoites are transformed into cysts under stress conditions *(Schaap & Schilde 2018)*. In *Acanthamoeba*, several pathways, for example, actin dynamics, glycolysis, proteolysis *(Bouyer et al. 2009)*, proteins such as cyst specific protein 21 *(Chen et al. 2004)*, serine protease *(Dudley et al. 2008; Moon et al. 2008)*, cysteine protease *(Leitsch et al. 2010; Moon et al. 2012)*, glycogen phosphorylase *(Lorenzo-Morales et al. 2008)*, sirtuin proteins *(Joo et al. 2020)*, and Shwachman-Bodian-Diamond syndrome protein *(Wang et al. 2021)* have been reported to be involved with this mechanism. However, coordination and crosstalk among these pathways to support the encystation are still unknown. Single or multiple pathways may be required for cysts formation, which probably depends on the strength and specificity of the encystment signal. Autophagy is an intracellular stress-sensing mechanism that occurs rapidly in response to stimuli such as rapamycin, starvation, or cytokines *(Kamada et al. 2004; Kroemer et al. 2010)*. So far, more than 30 autophagy-related (Atg) proteins have been

identified in yeast and humans, and their roles in this pathway have been extensively studied *(Feng et al. 2014; Galluzzi et al. 2017; Kamada et al. 2004)*. However, a partial list of Atg proteins has been characterized in *Acanthamoeba* i.e. Atg3 *(Moon et al. 2011)*, Atg8 *(Moon et al. 2009; Moon et al. 2013)*, Atg12 *(Kim et al. 2015)*, Atg16 *(Fujita et al. 2008)*. Altogether, our study thus analyzed the transcriptional expression of these genes upon curcumin treatment.

Starvation or a nutrient-depleted condition is a classical autophagy inducer in several eukaryotic cells *(Kamada et al. 2004; Mejlvang et al. 2018; Mizushima et al. 2004)*. In *Acanthamoeba*, starved conditions are able to induce *Acanthamoeba* encystation at different degrees depending on the media formulation, time, and *Acanthamoeba* spp. *(Aqeel et al. 2013; Boonhok et al. 2021a; Sohn et al. 2017)*. In our study, starvation by Page's Saline buffer (PAS) supplemented with 5% glucose was utilized. Approximately 40-50% of cysts were observed at 24h, and a percentage of trophozoites containing enlarged vacuoles was significantly increased. Autophagy inhibitors, 3MA, and wortmannin, which are known to inhibit phosphatidylinositol 3-kinase (PI3K) activity in the autophagy pathway *(Wu et al. 2010)*, were also applied to see an autophagic response in *A. triangularis*. The 3MA significantly inhibited *A. triangularis* cysts formation while wortmannin was slightly affected. The different degrees of inhibition may result from the specificity of binding to their PI3K substrate and the concentration used in the assay. In the presence of 50 µg/mL curcumin under starved conditions, a majority of the parasites remained in the trophozoites stage. Basically, induction of autophagy, a double membrane autophagosome or vacuole is formed *(Huang & Klionsky 2002; Nakatogawa et al. 2009)*, and in *Acanthamoeba*, formation of vacuoles including autophagosome and autolysosome is associated with the cyst wall formation *(Bowers & Korn 1969)*. The percentage of trophozoites containing vacuoles or enlarged vacuoles was thus analyzed by microscopy in our study. Due to a highly active trophozoites stage *(Alves et al. 2017)*, analysis of the trophozoites containing vacuoles, almost 100% of the trophozoites contained vacuoles, and the percentage of trophozoites containing vacuoles regardless of vacuole size did not make a difference among tested conditions. However, analysis of trophozoites containing enlarged vacuoles, the percentage was significantly reduced upon autophagy inhibitor or curcumin treatment. Moreover, a combination of curcumin with autophagy inhibitors did not completely inhibit cysts formation. To our drug combination study, no drug interaction was observed. Thus, this might indicate other intracellular pathways that are involved with *A. triangualris* encystation. Regarding curcumin's effect under the microscope that markedly inhibited cysts formation and increased vacuolization in the surviving trophozoites, molecular analysis of mRNA expression of ATG genes in the surviving trophozoites was performed to assess a physiological change in response to curcumin. Moon and his colleague firstly characterized Atg8 in *Acanthamoeba castellanii (Moon et al. 2009)*. *Ac*Atg8 was distributed in the amoeba cytosol, and its expression was peaked during encystation. In addition, intracellular colocalization of *Ac*Atg8 and lysosome on the membrane was demonstrated *(Moon et al. 2009)*. An *Ac*Atg8 isoform, *Ac*Atg8b, was later identified. This isoform was highly expressed during encystation and was required for *Acanthamoeba* encystation *(Moon et al. 2013)*. Atg3, an E2 ubiquitin-like conjugating enzyme, is

known to play a role in Atg8 conjugation system *(Feng et al. 2014)*. In *A. castellanii*. *Ac*Atg3 was investigated by Moon and his colleagues and found that its mRNA expression was not increased during the encystation, but the depletion of *Ac*Atg3 affected the maturation of cysts *(Moon et al. 2011)*. Atg12 plays a role in autophagosome formation by forming an Atg12-Atg5-Atg16L1 complex and acting as an E3-like enzyme for promoting Atg8 lipidation on the autophagosomal membrane *(Yin et al. 2016)*. At the early phase of encystation, *Acanthamoeba* Atg12 was consistently distributed in trophozoites. Later, it was formed as a puncta and colocalized with an autophagic membrane. Even its mRNA expression was not increased during encystation as expected, but it was crucial for the encystation as the down-regulation of *Ac*Atg12 in trophozoites inhibited cysts formation *(Kim et al. 2015)*. *Acanthamoeba* Atg16 was partially colocalized with autophagolysosome and highly expressed during *A. castellanii* encystation *(Song et al. 2012)*. Depletion of *Ac*Atg16 inhibited autophagosome formation and further disrupted the encystation mechanism *(Song et al. 2012)*. As expected, all tested genes were at the basal level upon 3MA- or curcumin-treated condition. The inhibition of key ATG mRNA expression thus supports the attenuation of *A. triangularis* encystation as well as cyst production. To these data, it is possible that the strength of curcumin signal is much stronger than starvation signal as autophagy is a tightly regulated pathway and its response depends on the strength and specificity of signals *(Kroemer et al. 2010; Simon et al. 2017)*, or curcumin might directly or indirectly interact with proteins associated with cell cycle which resulting in cell arrest at the trophozoites stage *(Bínová et al. 2021)*.

We next investigated the effect of a sole curcumin signal under a full condition or a nutrient-rich condition using PYG medium. The cyst formation in response to curcumin was at the basal level similar to that of full medium alone. The percentage of surviving trophozoites with enlarged vacuoles was also at the basal level and not different between curcumin-treated and untreated conditions. In addition, the real-time PCR analysis revealed that the tested ATG genes were similar to those of curcumin treatment under starved condition except *Ac*ATG16 that up-regulated at later time points. The increased expression of *Ac*ATG16 was also observed in *Peganum harmala* seed extract-treated *A. triangularis (Boonhok et al. 2021a)*; however, in *Cassia angustifolia* extract treatment, the increase of *Ac*ATG16 mRNA was not observed *(Boonhok et al. 2021b)*. It might indicate a role of Atg16 in *Acanthamoeba* in response to the specific stress signal in autophagy or other cellular pathways, which requires further investigations. Under the nutrient-rich condition, mRNA expression of other *A. triangularis* encystation-related genes was investigated. Both cellulose synthase (EDCBI66TR) and serine proteinase (EU365404) were slightly changed over the first 18h, and to our surprise, at 24h, their expression was significantly increased even the microscopic examination showed that there was no cyst induction at this time point. Cellulose is the main component of cyst wall, and three enzymes i.e. glycogen phosphorylase, UDP-glucose pyrophosphorylase, and cellulose synthase, are required for cellulose synthesis during *Acanthamoeba* encystation *(Garajová et al. 2019; Moon & Kong 2012)*. In addition to cellulose synthase, investigation on the mRNA expression of another two genes is required to predict cyst

formation after 24h conclusively; otherwise, this may indicate an additional function of the cellulose synthase. On the other hand, serine proteinase that increased at 24h post curcumin treatment may indicate its role in other cellular activities in addition to cell differentiation *(A Rascon & H McKerrow 2013; Blaschitz et al. 2006)*. Moreover, we observed metacaspase, which is known to be involved with apoptosis-like cell death in several microorganisms and associated with *A. castellanii* encystation *(Saheb et al. 2014; Trzyna et al. 2008)*, as well as interleukin-1 converting enzyme-like protease, known as caspase-1, has a role in programmed cell death of parasites *(Kosec et al. 2006; Wu et al. 2018)*. The mRNA expression of metacaspase (AF480890) was pretty consistent over the time period of curcumin treatment which may support no cyst formation. However, interleukin-1 converting enzyme-like protease (XM_004338552) was a quick response to curcumin as its mRNA expression was immediately increased at 6h post-treatment. However, at later time points, its expression was declined to the basal level. The increase of this gene at an early time point may indicate an apoptotic cell death by curcumin. However, to confirm this type of cell death, an apoptosis assay is required. Once the amoeba can cope with the curcumin stress, the interleukin-1 converting enzyme-like protease expression is gradually declined, which reveals an ability of *A. triangularis* trophozoites to overcome the curcumin stress or a death signal.

To our results that demonstrated *A. triangularis* arrest at the trophozoites stage by curcumin, the underlying mechanism, whether curcumin is directly involved in autophagy or cell cycle pathway or there is a connection between these two pathways, is still unknown and needs further investigations. In the line of curcumin effect on autophagy, curcumin is known to modulate autophagy*(Shakeri et al. 2019)*, and the outcome is varied depending on cell type and curcumin concentration as described herein. In human endothelial cells, EA.hy926 and HUVECs, 5 or 20 μM curcumin induced autophagy in order to reduce oxidative stress-induced cell damage *(Guo et al. 2016; Han et al. 2012)*. Curcumin of 40 μM was able to induce autophagy which is partially involved with anticancer activity in human lung adenocarcinoma cell line, A549 *(Liu et al. 2017)*. In human colon cancer cells, HCT116, 40 μM curcumin-induced reactive oxygen species (ROS) production, which further activated autophagy followed by cell death *(Liu et al. 2017)*. On the other hand, in mouse hippocampal neuronal cell line, HT-22, 10 or 15 μM curcumin promoted cell recovery in Aβ1-42-treated condition by inhibiting autophagy *(Zhang et al. 2018)*. At 5 μM curcumin, it reduced apoptosis cell death and inhibited autophagy and hypoxia-inducible factor 1-alpha in rat adrenal pheochromocytoma cell, PC12, model of oxygen-glucose deprivation/reperfusion (OGD/R) condition *(Hou et al. 2019)*. Along with the OGD/R model, 10 μM curcumin was able to increase the resistance of cortical neurons by reducing autophagy and cell apoptosis in an mTOR-dependent manner *(Shi et al. 2019)*. In H9c2 myocytes, curcumin demonstrated a protective effect against hypoxia/reoxygenation (H/R) injury through the inhibition of apoptosis and autophagy *(Huang et al. 2015)*. Regarding curcumin's ability on cell arrest, several studies have mentioned on this pharmacological activity. Curcumin treatment caused cell cycle arrest at G1/S and G2/M phases and activated a caspase-3 pathway, resulting in human osteosarcoma (HOS) cell death *(Lee et al. 2009)*. In human cervical carcinoma cells, SiHa cells,

curcumin activated ROS production, apoptosis, autophagy, cell cycle arrest, and cellular senescence. These activities co-occurred with the upregulation of p53 and p21 proteins *(Wang & Wu 2020)*. In colon cancer cells, HT-29, curcumin-induced ROS production led to apoptotic cell death and cell cycle inhibition *(Agarwal et al. 2018)*. In pancreatic cancer cells, BxPC-3, curcumin treatment increased phosphorylation of an Ataxia-telangiectasia mutated kinase and checkpoint kinase-1 proteins and then mediated G2/M cell cycle arrest and apoptosis *(Sahu et al. 2009)*. However, the study in parasites including amoeba, an amoebicidal activity *(El-Sayed et al. 2012; Mungroo et al. 2020; Rangel-Castañeda et al. 2018)*, anti-virulence *(Rangel-Castañeda et al. 2019)*, anti-adhesion *(Mitsuwan et al. 2020b)*, and effect on cell physiology *(Swatson et al. 2017)* by curcumin was reported. Even autophagy is a quick response to various stimuli, but its mechanism is tightly regulated and be more selective in which Atg proteins work together in a specific manner and coordinate with other pathways or proteins to create a wide variety of physiological processes in cells *(Galluzzi et al. 2017; Wang & Qin 2013)*. Investigation of function and physiological change of *A. triangularis* Atg proteins in response to stresses, including the curcumin stress, is needed.

Curcumin and curcumin derivatives have been extensively studied so far for therapeutic purposes, especially in parasitic infections *(Din et al. 2016)*. A successful development of a new class of curcumin has been reported against *Trypanosoma cruzi (Matiadis et al. 2021)*. In *Plasmodium* infection, several strategies have been developed to increase the effectiveness of curcumin, for example, nanotized curcumin formulation *(Ghosh et al. 2014)* and curcumin containing liposomes *(Martí Coma-Cros et al. 2018)*. These are to improve solubility, target selectivity, and reduce the frequency of administration. The strategies open another direction on drug development that could be applied in *Acanthamoeba* research. Moreover, drug combination strategy by targeting autophagy pathway in other models has been reported *(Zanotto-Filho et al. 2015)*, and this strategy may be possible to apply in *Acanthamoeba* infection in the future. Taken together, in addition to the evaluation of the risk of *Acanthamoeba* encystation through autophagy under the drug or natural compound pressure, the drug combination study is of our interest as it has been shown to improve therapeutic efficacy and help reduce the drug resistance cycle in infectious diseases *(Hill & Cowen 2015)*.

Conclusions

Curcumin contains a wide range of pharmacological activities and medicinal properties against numerous diseases. In *Acanthamoeba* infection, the amoebicidal activity of curcumin is recently discovered. However, the effect of curcumin on the surviving *A. triangularis* trophozoites has never been reported. Our study provides information on the surviving *A. triangularis* response to curcumin which results in *A. triangularis* arrest at the trophozoites stage, not transforming to the cyst, even under starvation, which is used to induce cysts formation, or in a nutrient-rich condition. The physiological change of autophagy at the transcriptional level is slightly changed. The

presence of *A. triangularis* trophozoites under curcumin stress is a good indication in terms of *Acanthamoeba* treatment as it is more susceptible to drug/compound than cysts form. In addition, no upregulation of autophagy-related genes indicates a less likely to induce *A. triangularis* encystation. Altogether, a dual benefit of curcumin, amoebicidal activity, and arresting cysts transformation could be another evidence to support curcumin development and future use of curcumin in *Acanthamoeba* infection therapy. However, further investigations on the role of *Acanthamoeba* autophagy proteins in response to stress are needed to understand the stress response mechanism, coordination with other cellular pathways, and their association with *Acanthamoeba* encystation.

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References

A Rascon A, and H McKerrow J. 2013. Synthetic and natural protease inhibitors provide insights into parasite development, virulence and pathogenesis. *Current medicinal chemistry* 20:3078-3102.

Abjani F, Khan NA, Yousuf FA, and Siddiqui R. 2016. Targeting cyst wall is an effective strategy in improving the efficacy of marketed contact lens disinfecting solutions against *Acanthamoeba castellanii* cysts. *Contact Lens and Anterior Eye* 39:239-243.

Agarwal A, Kasinathan A, Ganesan R, Balasubramanian A, Bhaskaran J, Suresh S, Srinivasan R, Aravind K, and Sivalingam N. 2018. Curcumin induces apoptosis and cell cycle arrest via the activation of reactive oxygen species–independent mitochondrial apoptotic pathway in Smad4 and p53 mutated colon adenocarcinoma HT29 cells. *Nutrition Research* 51:67-81.

Alves DdSMM, Alves LM, da Costa TL, de Castro AM, and Vinaud MC. 2017. Anaerobic metabolism in T4 *Acanthamoeba* genotype. *Current Microbiology* 74:685-690.

Anwar A, Khan NA, and Siddiqui R. 2018. Combating *Acanthamoeba* spp. cysts: what are the options? *Parasite Vector* 11:26.

Aqeel Y, Siddiqui R, Iftikhar H, and Khan NA. 2013. The effect of different environmental conditions on the encystation of *Acanthamoeba castellanii* belonging to the T4 genotype. *Exp Parasitol* 135:30-35.

Bínová E, Bína D, and Nohýnková E. 2021. DNA content in *Acanthamoeba* during two stress defense reactions: Encystation, pseudocyst formation and cell cycle. *European Journal of Protistology* 77:125745.

Blaschitz M, Köhsler M, Aspöck H, and Walochnik J. 2006. Detection of a serine proteinase gene in *Acanthamoeba* genotype T6 (Amoebozoa: Lobosea). *Experimental parasitology* 114:26-33.

Boonhok R, Sangkanu S, Chuprom J, Srisuphanunt M, Norouzi R, Siyadatpanah A, Mirzaei F, Mitsuwan W, Wisessombat S, and de Lourdes Pereira M. 2021a. *Peganum harmala* Extract Has Antiamoebic Activity to *Acanthamoeba triangularis* Trophozoites and Changes Expression of Autophagy-Related Genes. *Pathogens* 10:842.

Boonhok R, Sangkanu S, Norouzi R, Siyadatpanah A, Mirzaei F, Mitsuwan W, Charong N, Wisessombat S, de Lourdes Pereira M, and Rahmatullah M. 2021b. Amoebicidal activity of *Cassia angustifolia* extract and its effect on *Acanthamoeba triangularis* autophagy-related gene expression at transcriptional level. *Parasitology*:1-36.

Bouyer S, Rodier M-H, Guillot A, and Héchard Y. 2009. *Acanthamoeba castellanii*: proteins involved in actin dynamics, glycolysis, and proteolysis are regulated during encystation. *Exp Parasitol* 123:90-94.

Bowers B, and Korn ED. 1969. The fine structure of *Acanthamoeba castellanii* (Neff strain) II. Encystment. *The Journal of cell biology* 41:786-805.

Brinck Andersen N-S, Jorgensen SE, Skipper KA, Larsen SM, Heinz J, Thomsen MM, Farahani E, Cai Y, Hait AS, and Kay L. 2020. Essential role of autophagy in restricting poliovirus infection revealed by identification of an ATG7 defect in a poliomyelitis patient. *Autophagy*:1-16.

Castillo EF, Dekonenko A, Arko-Mensah J, Mandell MA, Dupont N, Jiang S, Delgado-Vargas M, Timmins GS, Bhattacharya D, and Yang H. 2012. Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proceedings of the National Academy of Sciences* 109:E3168-E3176.

Cervantes-Valencia ME, Hermosilla C, Alcalá-Canto Y, Tapia G, Taubert A, and Silva LM. 2019. Antiparasitic efficacy of curcumin against *Besnoitia besnoiti* tachyzoites *in vitro*. *Frontiers in veterinary science* 5:333.

Chelkha N, Jardot P, Moussaoui I, Levasseur A, La Scola B, and Colson P. 2020. Core gene-based molecular detection and identification of *Acanthamoeba* species. *Scientific reports* 10:1-9.

Chen L, Orfeo T, Gilmartin G, and Bateman E. 2004. Mechanism of cyst specific protein 21 mRNA induction during Acanthamoeba differentiation. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1691:23-31.

Cui L, Miao J, and Cui L. 2007. Cytotoxic effect of curcumin on malaria parasite *Plasmodium falciparum*: inhibition of histone acetylation and generation of reactive oxygen species. *Antimicrobial agents and chemotherapy* 51:488-494.

de Lacerda AG, and Lira M. 2021. *Acanthamoeba* keratitis: a review of biology, pathophysiology and epidemiology. *Ophthalmic Physiol Opt* 41:116-135.

de Paula Aguiar D, Brunetto Moreira Moscardini M, Rezende Morais E, Graciano de Paula R, Ferreira PM, Afonso A, Belo S, Tomie Ouchida A, Curti C, and Cunha WR. 2016. Curcumin generates oxidative stress and induces apoptosis in adult *Schistosoma mansoni* worms. *PloS one* 11:e0167135.

Díaz-Troya S, Pérez-Pérez ME, Florencio FJ, and Crespo JL. 2008. The role of TOR in autophagy regulation from yeast to plants and mammals. *Autophagy* 4:851-865.

Din ZU, dos Santos A, Trapp MA, Lazarin-Bidóia D, Garcia FP, Peron F, Nakamura CV, and Rodrigues-Filho E. 2016. Curcumin inspired synthesis of unsymmetrical diarylpentanoids with highly potent anti-parasitic activities: *in silico* studies and DFT-based stereochemical calculation. *MedChemComm* 7:820-831.

Dudley R, Alsam S, and Khan NA. 2008. The role of proteases in the differentiation of *Acanthamoeba castellanii*. *FEMS Microbiology Letters* 286:9-15.

El-Sayed NM, Ismail KA, Ahmed SA-E-G, and Hetta MH. 2012. *In vitro* amoebicidal activity of ethanol extracts of *Arachis hypogaea* L., *Curcuma longa* L. and *Pancratium maritimum* L. on *Acanthamoeba castellanii* cysts. *Parasitology Research* 110:1985-1992.

Elsheikha HM, Siddiqui R, and Khan NA. 2020. Drug discovery against *Acanthamoeba* infections: Present knowledge and unmet needs. *Pathogens* 9:405.

Eskelinen E-L. 2005. Maturation of autophagic vacuoles in mammalian cells. *Autophagy* 1:1-10.

Feng Y, He D, Yao Z, and Klionsky DJ. 2014. The machinery of macroautophagy. *Cell Res* 24:24-41.

Fujita N, Itoh T, Omori H, Fukuda M, Noda T, and Yoshimori T. 2008. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Molecular biology of the cell* 19:2092-2100.

Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, Choi AM, Chu CT, Codogno P, and Colombo MI. 2017. Molecular definitions of autophagy and related processes. *The EMBO journal* 36:1811-1836.

Garajová M, Mrva M, Vaškovicová N, Martinka M, Melicherová J, and Valigurová A. 2019. Cellulose fibrils formation and organisation of cytoskeleton during encystment are essential for *Acanthamoeba* cyst wall architecture. *Sci Rep* 9:1-21.

Ghosh A, Banerjee T, Bhandary S, and Surolia A. 2014. Formulation of nanotized curcumin and demonstration of its antimalarial efficacy. *International journal of nanomedicine* 9:5373.

675 Guimaraes AJ, Gomes KX, Cortines JR, Peralta JM, and Peralta RHS. 2016. *Acanthamoeba* spp.
676 as a universal host for pathogenic microorganisms: One bridge from environment to host
677 virulence. *Microbiological Research* 193:30-38.
678 Guo S, Long M, Li X, Zhu S, Zhang M, and Yang Z. 2016. Curcumin activates autophagy and
679 attenuates oxidative damage in EA. hy926 cells via the Akt/mTOR pathway. *Molecular*
680 *medicine reports* 13:2187-2193.
681 Gutiérrez-Gutiérrez F, Palomo-Ligas L, Hernández-Hernández JM, Pérez-Rangel A, Aguayo-
682 Ortiz R, Hernández-Campos A, Castillo R, González-Pozos S, Cortés-Zárate R, and
683 Ramírez-Herrera MA. 2017. Curcumin alters the cytoskeleton and microtubule
684 organization on trophozoites of *Giardia lamblia*. *Acta Tropica* 172:113-121.
685 Han J, Pan X-Y, Xu Y, Xiao Y, An Y, Tie L, Pan Y, and Li X-J. 2012. Curcumin induces
686 autophagy to protect vascular endothelial cell survival from oxidative stress damage.
687 *Autophagy* 8:812-825.
688 Hill JA, and Cowen LE. 2015. Using combination therapy to thwart drug resistance. *Future*
689 *Microbiology* 10:1719-1726.
690 Hou Y, Wang J, and Feng J. 2019. The neuroprotective effects of curcumin are associated with the
691 regulation of the reciprocal function between autophagy and HIF-1α in cerebral ischemia-
692 reperfusion injury. *Drug Design, Development and Therapy* 13:1135.
693 Huang W-P, and Klionsky DJ. 2002. Autophagy in yeast: a review of the molecular machinery.
694 *Cell Structure and Function* 27:409-420.
695 Huang Z, Ye B, Dai Z, Wu X, Lu Z, Shan P, and Huang W. 2015. Curcumin inhibits autophagy
696 and apoptosis in hypoxia/reoxygenation-induced myocytes. *Molecular medicine reports*
697 11:4678-4684.
698 Hussain RHM, Isa NSM, Kamaruddin KA, Ghani MKA, Khan NA, Siddiqui R, and Anuar TS.
699 2020. Morphological and molecular characterization of *Acanthamoeba* isolated from
700 contact lens paraphernalia in Malaysia: Highlighting the pathogenic potential of T4
701 genotype. *Asian Pacific Journal of Tropical Medicine* 13:542.
702 Hussein A, Rashed S, El Hayawan I, El-Sayed R, and Ali H. 2017. Evaluation of the anti-
703 schistosomal effects of turmeric (*Curcuma longa*) versus praziquantel in *Schistosoma*
704 *mansoni* infected mice. *Iranian journal of parasitology* 12:587.
705 Jakubczyk K, Drużga A, Katarzyna J, and Skonieczna-Żydecka K. 2020. Antioxidant Potential of
706 Curcumin—A Meta-Analysis of Randomized Clinical Trials. *Antioxidants* 9:1092.
707 Joo S-Y, Aung JM, Shin M, Moon E-K, Kong H-H, Goo Y-K, Chung D-I, and Hong Y. 2020. The
708 role of the *Acanthamoeba castellanii* Sir2-like protein in the growth and encystation of
709 *Acanthamoeba*. *Parasites & vectors* 13:1-14.
710 Juarez MM, Tártara LI, Cid AG, Real JP, Bermúdez JM, Rajal VB, and Palma SD. 2018.
711 *Acanthamoeba* in the eye, can the parasite hide even more? Latest developments on the
712 disease. *Cont Lens Anterior Eye* 41:245-251.
713 Kamada Y, Sekito T, and Ohsumi Y. 2004. Autophagy in yeast: ATOR-mediated response to
714 nutrient starvation. *TOR*:73-84.
715 Khambu B, Yan S, Huda N, Liu G, and Yin X-M. 2018. Autophagy in non-alcoholic fatty liver
716 disease and alcoholic liver disease. *Liver Res* 2:112-119.
717 Khan NA, Anwar A, and Siddiqui R. 2019. *Acanthamoeba* keratitis: current status and urgent
718 research priorities. *Curr Med Chem* 26:5711-5726.
719 Kim S-H, Moon E-K, Hong Y, Chung D-I, and Kong H-H. 2015. Autophagy protein 12 plays an
720 essential role in *Acanthamoeba* encystation. *Experimental parasitology* 159:46-52.

Kim SY, Syms MJ, Holtel MR, and Nauschuetz KK. 2000. *Acanthamoeba* sinusitis with subsequent dissemination in an AIDS patient. *Ear, nose & throat journal* 79:168-174.

Kocaadam B, and Şanlier N. 2017. Curcumin, an active component of turmeric (*Curcuma longa*), and its effects on health. *Critical Reviews in Food Science and Nutrition* 57:2889-2895.

Koide T, Nose M, Ogihara Y, Yabu Y, and Ohta N. 2002. Leishmanicidal effect of curcumin i*n vitro*. *Biological and Pharmaceutical Bulletin* 25:131-133.

Kosec G, Alvarez VE, Agüero F, Sánchez D, Dolinar M, Turk B, Turk V, and Cazzulo JJ. 2006. Metacaspases of *Trypanosoma cruzi*: possible candidates for programmed cell death mediators. *Molecular and biochemical parasitology* 145:18-28.

Kroemer G, Mariño G, and Levine B. 2010. Autophagy and the integrated stress response. *Molecular cell* 40:280-293.

Lee DS, Lee MK, and Kim JH. 2009. Curcumin induces cell cycle arrest and apoptosis in human osteosarcoma (HOS) cells. *Anticancer Research* 29:5039-5044.

Leitsch D, Köhsler M, Marchetti-Deschmann M, Deutsch A, Allmaier G, Duchêne M, and Walochnik J. 2010. Major role for cysteine proteases during the early phase of *Acanthamoeba castellanii* encystment. *Eukaryotic Cell* 9:611-618.

Liu F, Gao S, Yang Y, Zhao X, Fan Y, Ma W, Yang D, Yang A, and Yu Y. 2017. Curcumin induced autophagy anticancer effects on human lung adenocarcinoma cell line A549. *Oncology Letters* 14:2775-2782.

Lorenzo-Morales J, Khan NA, and Walochnik J. 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite* 22.

Lorenzo-Morales J, Kliescikova J, Martinez-Carretero E, De Pablos LM, Profotova B, Nohynkova E, Osuna A, and Valladares B. 2008. Glycogen phosphorylase in *Acanthamoeba* spp.: determining the role of the enzyme during the encystment process using RNA interference. *Eukaryotic Cell* 7:509-517.

Martí Coma-Cros E, Biosca A, Lantero E, Manca ML, Caddeo C, Gutiérrez L, Ramírez M, Borgheti-Cardoso LN, Manconi M, and Fernàndez-Busquets X. 2018. Antimalarial activity of orally administered curcumin incorporated in Eudragit®-containing liposomes. *International journal of molecular sciences* 19:1361.

Martins C, Da Silva D, Neres A, Magalhaes T, Watanabe G, Modolo L, Sabino A, De Fátima A, and De Resende M. 2009. Curcumin as a promising antifungal of clinical interest. *Journal of Antimicrobial Chemotherapy* 63:337-339.

Matiadis D, Saporiti T, Aguilera E, Robert X, Guillon C, Cabrera N, Pérez-Montfort R, Sagnou M, and Alvarez G. 2021. Pyrazol (in) e derivatives of curcumin analogs as a new class of anti-*Trypanosoma cruzi* agents. *Future Medicinal Chemistry* 13:701-714.

Matson DO, Rouah E, Lee RT, Armstrong D, Parke JT, and Baker CJ. 1988. *Acanthamoeba* meningoencephalitis masquerading as neurocysticercosis. *The Pediatric infectious disease journal* 7:121-124.

Mejlvang J, Olsvik H, Svenning S, Bruun J-A, Abudu YP, Larsen KB, Brech A, Hansen TE, Brenne H, and Hansen T. 2018. Starvation induces rapid degradation of selective autophagy receptors by endosomal microautophagy. *Journal of Cell Biology* 217:3640-3655.

Menzies FM, Fleming A, and Rubinsztein DC. 2015. Compromised autophagy and neurodegenerative diseases. *Nature Reviews Neuroscience* 16:345-357.

765 Mishra S, Karmodiya K, Surolia N, and Surolia A. 2008. Synthesis and exploration of novel
766 curcumin analogues as anti-malarial agents. *Bioorganic & medicinal chemistry* 16:2894-
767 2902.
768 Mitsuwan W, Bunsuwansakul C, Leonard TE, Laohaprapanon S, Hounkong K, Bunluepuech K,
769 Chalermpol K, Mahboob T, Sumudi Raju C, and Dhobi M. 2020a. *Curcuma longa* ethanol
770 extract and Curcumin inhibit the growth of *Acanthamoeba triangularis* trophozoites and
771 cysts isolated from water reservoirs at Walailak University, Thailand. *Pathogens and*
772 *Global Health* 114:1-11.
773 Mitsuwan W, Sangkanu S, Romyasamit C, Kaewjai C, Jimoh TO, de Lourdes Pereira M,
774 Siyadatpanah A, Kayesth S, Nawaz M, and Rahmatullah M. 2020b. *Curcuma longa*
775 rhizome extract and Curcumin reduce the adhesion of *Acanthamoeba triangularis*
776 trophozoites and cysts in polystyrene plastic surface and contact lens. *International Journal*
777 *for Parasitology: Drugs and Drug Resistance* 14:218-229.
778 Mizushima N, Yamamoto A, Matsui M, Yoshimori T, and Ohsumi Y. 2004. *In vivo* analysis of
779 autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent
780 autophagosome marker. *Molecular biology of the cell* 15:1101-1111.
781 Moon E-K, Chung D-I, Hong Y-C, and Kong H-H. 2008. Characterization of a serine proteinase
782 mediating encystation of *Acanthamoeba*. *Eukaryotic Cell* 7:1513-1517.
783 Moon E-K, Chung D-I, Hong Y-C, and Kong H-H. 2009. Autophagy protein 8 mediating
784 autophagosome in encysting *Acanthamoeba*. *Molecular and biochemical parasitology*
785 168:43-48.
786 Moon E-K, Chung D-I, Hong Y, and Kong H-H. 2011. Atg3-mediated lipidation of Atg8 is
787 involved in encystation of *Acanthamoeba*. *The Korean journal of parasitology* 49:103.
788 Moon E-K, Chung D-I, Hong Y, and Kong H-H. 2012. Protein kinase C signaling molecules
789 regulate encystation of *Acanthamoeba*. *Experimental parasitology* 132:524-529.
790 Moon E-K, Hong Y, Chung D-I, and Kong H-H. 2013. Identification of atg8 isoform in encysting
791 *Acanthamoeba*. *The Korean journal of parasitology* 51:497.
792 Moon E-K, and Kong H-H. 2012. Short-cut pathway to synthesize cellulose of encysting
793 *Acanthamoeba*. *The Korean journal of parasitology* 50:361.
794 Morrison AO, Morris R, Shannon A, Lauer SR, Guarner J, and Kraft CS. 2016. Disseminated
795 *Acanthamoeba* infection presenting with cutaneous lesions in an immunocompromised
796 patient: a case report, review of histomorphologic findings, and potential diagnostic
797 pitfalls. *American journal of clinical pathology* 145:266-270.
798 Mungroo MR, Anwar A, Khan NA, and Siddiqui R. 2020. Gold-conjugated curcumin as a novel
799 therapeutic agent against brain-eating amoebae. *ACS omega* 5:12467-12475.
800 Nakatogawa H, Suzuki K, Kamada Y, and Ohsumi Y. 2009. Dynamics and diversity in autophagy
801 mechanisms: lessons from yeast. *Nature Reviews Molecular Cell Biology* 10:458-467.
802 Neelam S, and Niederkorn JY. 2017. Focus: infectious diseases: pathobiology and immunobiology
803 of *Acanthamoeba* keratitis: insights from animal models. *The Yale journal of biology and*
804 *medicine* 90:261.
805 Novaes RD, Sartini MVP, Rodrigues JPF, Gonçalves RV, Santos EC, Souza RLM, and Caldas IS.
806 2016. Curcumin enhances the anti-*Trypanosoma cruzi* activity of benznidazole-based
807 chemotherapy in acute experimental Chagas disease. *Antimicrobial agents and*
808 *chemotherapy* 60:3355-3364.

Picazarri K, Nakada-Tsukui K, and Nozaki T. 2008. Autophagy during proliferation and encystation in the protozoan parasite *Entamoeba invadens*. *Infection and immunity* 76:278-288.

Rangel-Castañeda IA, Carranza-Rosales P, Guzmán-Delgado NE, Hernández-Hernández JM, González-Pozos S, Pérez-Rangel A, and Castillo-Romero A. 2019. Curcumin attenuates the pathogenicity of *Entamoeba histolytica* by regulating the expression of virulence factors in an *ex-vivo* model infection. *Pathogens* 8:127.

Rangel-Castañeda IA, Hernández-Hernández JM, Pérez-Rangel A, González-Pozos S, Carranza-Rosales P, Charles-Niño CL, Tapia-Pastrana G, Ramírez-Herrera MA, and Castillo-Romero A. 2018. Amoebicidal activity of curcumin on *Entamoeba histolytica* trophozoites. *Journal of Pharmacy and Pharmacology* 70:426-433.

Rayamajhee B, Subedi D, Peguda HK, Willcox MD, Henriquez FL, and Carnt N. 2021. A Systematic Review of Intracellular Microorganisms within *Acanthamoeba* to Understand Potential Impact for Infection. *Pathogens* 10:225.

Saheb E, Trzyna W, and Bush J. 2014. Caspase-like proteins: *Acanthamoeba castellanii* metacaspase and *Dictyostelium discoideum* paracaspase, what are their functions? *Journal of biosciences* 39:909-916.

Sahu R, Batra S, and Srivastava S. 2009. Activation of ATM/Chk1 by curcumin causes cell cycle arrest and apoptosis in human pancreatic cancer cells. *British Journal of Cancer* 100:1425-1433.

Schaap P, and Schilde C. 2018. Encystation: the most prevalent and underinvestigated differentiation pathway of eukaryotes. *Microbiology* 164:727-739.

Shakeri A, Cicero AF, Panahi Y, Mohajeri M, and Sahebkar A. 2019. Curcumin: A naturally occurring autophagy modulator. *Journal of cellular physiology* 234:5643-5654.

Shi Q, Zhang Q, Peng Y, Zhang X, Wang Y, and Shi L. 2019. A natural diarylheptanoid protects cortical neurons against oxygen–glucose deprivation-induced autophagy and apoptosis. *Journal of Pharmacy and Pharmacology* 71:1110-1118.

Siddiqui R, and Khan NA. 2012. Biology and pathogenesis of *Acanthamoeba*. *Parasites & vectors* 5:6.

Simon H-U, Friis R, Tait SW, and Ryan KM. 2017. Retrograde signaling from autophagy modulates stress responses. *Science signaling* 10:eaag2791.

Sohn H-J, Kang H, Seo G-E, Kim J-H, Jung S-Y, and Shin H-J. 2017. Efficient liquid media for encystation of pathogenic free-living amoebae. *The Korean journal of parasitology* 55:233.

Song S-M, Han B-I, Moon E-K, Lee Y-R, Yu HS, Jha BK, Danne D-BS, Kong H-H, Chung D-I, and Hong Y. 2012. Autophagy protein 16-mediated autophagy is required for the encystation of *Acanthamoeba castellanii*. *Molecular and biochemical parasitology* 183:158-165.

Suzuki K. 2013. Selective autophagy in budding yeast. *Cell death & differentiation* 20:43-48.

Swatson WS, Katoh-Kurasawa M, Shaulsky G, and Alexander S. 2017. Curcumin affects gene expression and reactive oxygen species via a PKA dependent mechanism in *Dictyostelium discoideum*. *PloS one* 12:e0187562.

Taravaud A, Loiseau PM, and Pomel S. 2017. *In vitro* evaluation of antimicrobial agents on *Acanthamoeba* sp. and evidence of a natural resilience to amphotericin B. *The International Journal for Parasitology: Drugs and Drug Resistance* 7:328-336.

Teow S-Y, Liew K, Ali SA, Khoo AS-B, and Peh S-C. 2016. Antibacterial action of curcumin against *Staphylococcus aureus*: a brief review. *Journal of tropical medicine* 2016.

Tomeh MA, Hadianamrei R, and Zhao X. 2019. A review of curcumin and its derivatives as anticancer agents. *International journal of molecular sciences* 20:1033.

Trzyna WC, Legras XD, and Cordingley JS. 2008. A type-1 metacaspase from *Acanthamoeba castellanii*. *Microbiological Research* 163:414-423.

Turner N, Russell A, Furr J, and Lloyd D. 2000. Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *Journal of Antimicrobial Chemotherapy* 46:27-34.

Vallianou NG, Evangelopoulos A, Schizas N, and Kazazis C. 2015. Potential anticancer properties and mechanisms of action of curcumin. *Anticancer Research* 35:645-651.

Wal P, Saraswat N, Pal RS, Wal A, and Chaubey M. 2019. A detailed insight of the anti-inflammatory effects of curcumin with the assessment of parameters, sources of ros and associated mechanisms. *Open Medicine Journal* 6:64-76.

Wang T, and Wu X. 2020. Curcumin induces G2/M arrest and triggers autophagy, ROS generation and cell senescence in cervical cancer cells. *Journal of Cancer* 11:6704.

Wang Y-J, Lin W-C, and He M-S. 2021. The *Acanthamoeba* SBDS, a cytoskeleton-associated gene, is highly expressed during phagocytosis and encystation. *Journal of microbiology, immunology and infection* 54:482-489.

Wang Y, and Qin Z-h. 2013. Coordination of autophagy with other cellular activities. *Acta Pharmacologica Sinica* 34:585-594.

Wu D, Qiao K, Feng M, Fu Y, Cai J, Deng Y, Tachibana H, and Cheng X. 2018. Apoptosis of *Acanthamoeba castellanii* trophozoites induced by oleic acid. *Journal of Eukaryotic Microbiology* 65:191-199.

Wu Y-T, Tan H-L, Shui G, Bauvy C, Huang Q, Wenk MR, Ong C-N, Codogno P, and Shen H-M. 2010. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *Journal of Biological Chemistry* 285:10850-10861.

Yang M, Lee G, Si J, Lee S-J, You HJ, and Ko G. 2016. Curcumin shows antiviral properties against norovirus. *Molecules* 21:1401.

Yin Z, Pascual C, and Klionsky DJ. 2016. Autophagy: machinery and regulation. *Microbial cell* 3:588.

Yorimitsu T, and Klionsky DJ. 2005. Autophagy: molecular machinery for self-eating. *Cell death and differentiation* 12:1542-1552.

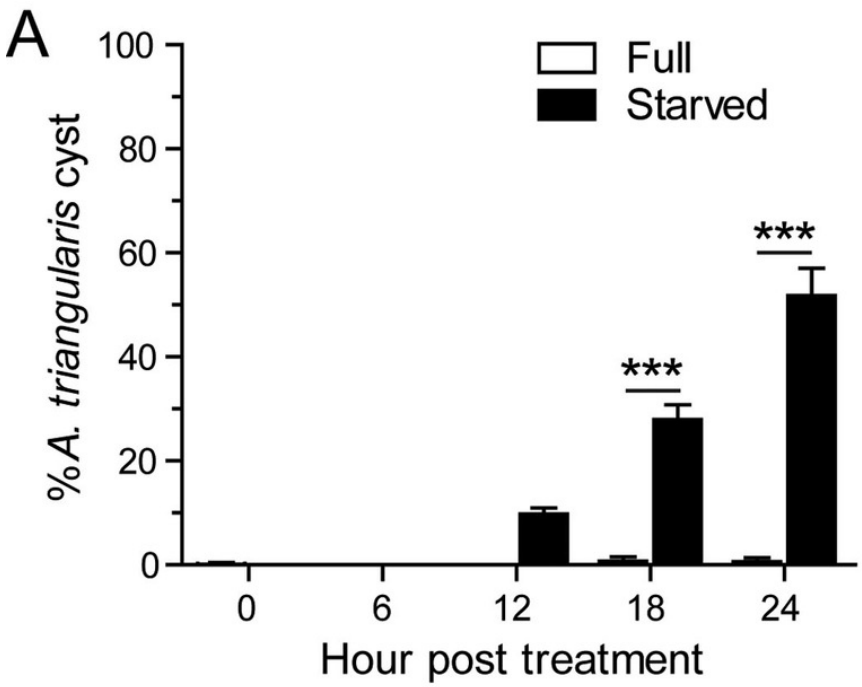
Zanotto-Filho A, Braganhol E, Klafke K, Figueiró F, Terra SR, Paludo FJ, Morrone M, Bristot IJ, Battastini AM, and Forcelini CM. 2015. Autophagy inhibition improves the efficacy of curcumin/temozolomide combination therapy in glioblastomas. *Cancer Letters* 358:220-231.

Zhang L, Fang Y, Cheng X, Lian Y, Zeng Z, Wu C, Zhu H, and Xu H. 2018. The potential protective effect of curcumin on amyloid-β-42 induced cytotoxicity in HT-22 cells. *BioMed Research International* 2018.

Figure 1

A. triangularis cysts formation under starvation.

(A) The trophozoites were cultured in the starvation medium, PAS supplemented with 5% glucose, for 24 h. The parasites grew in PYG medium, or full medium was used as a control. The parasites were stained with Trypan Blue, and the viable parasites were analyzed under a microscope at the indicated time points. *A. triangularis* cysts were counted and represented as mean percentage±SD. Data was obtained from 3 independent experiments. ***, $P<0.001$. **(B)** Representative images of parasites cultured in full and starved medium. Bar 20 μM. White and black arrowheads indicate the irregular trophozoites and rounded trophozoites, respectively, while the unfilled arrowhead indicates cysts.


A
100
80
60
40
20
0
%A. triangularis cyst
Full
Starved
***
***
0
6
12
18
24
Hour post treatment

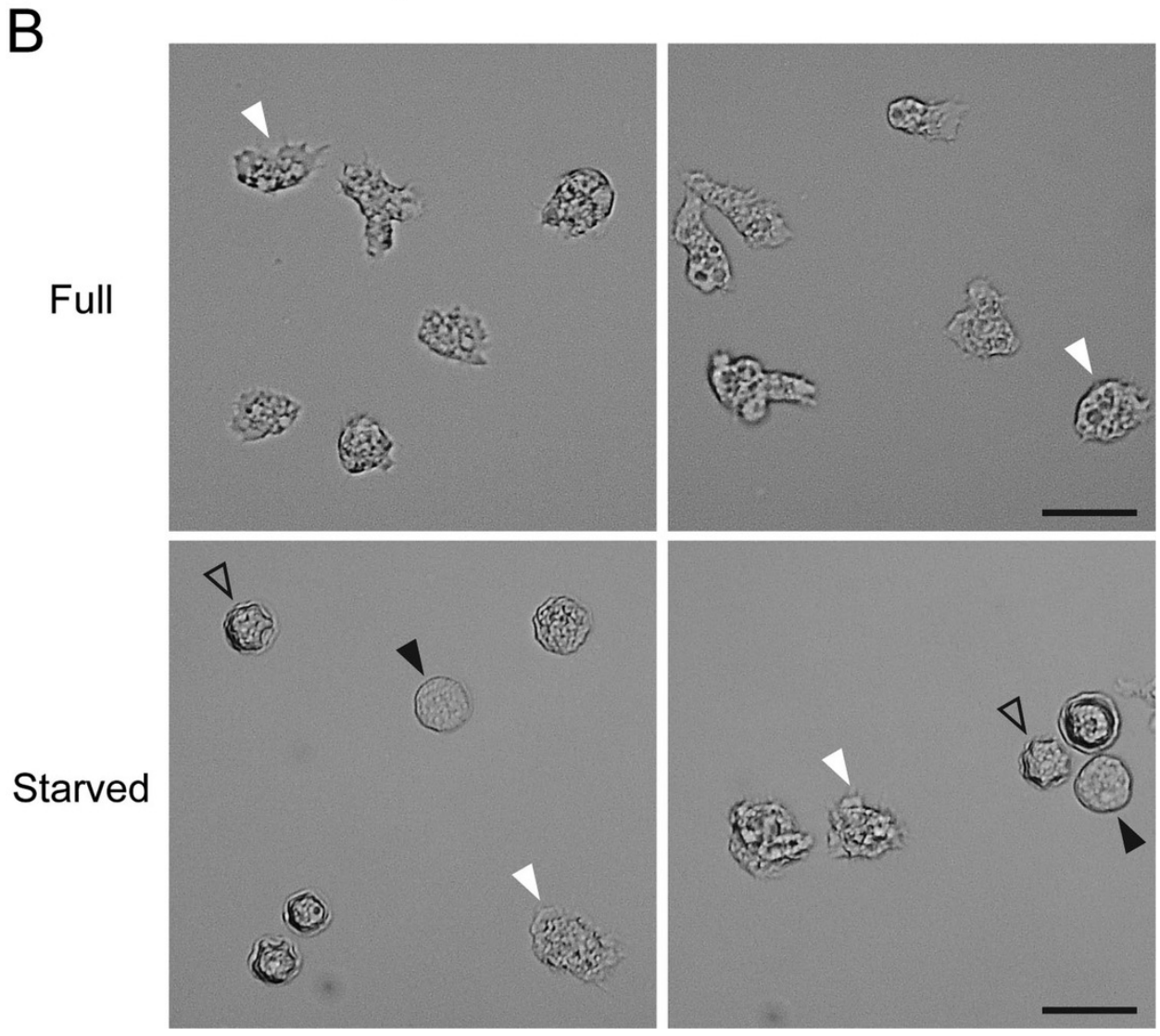
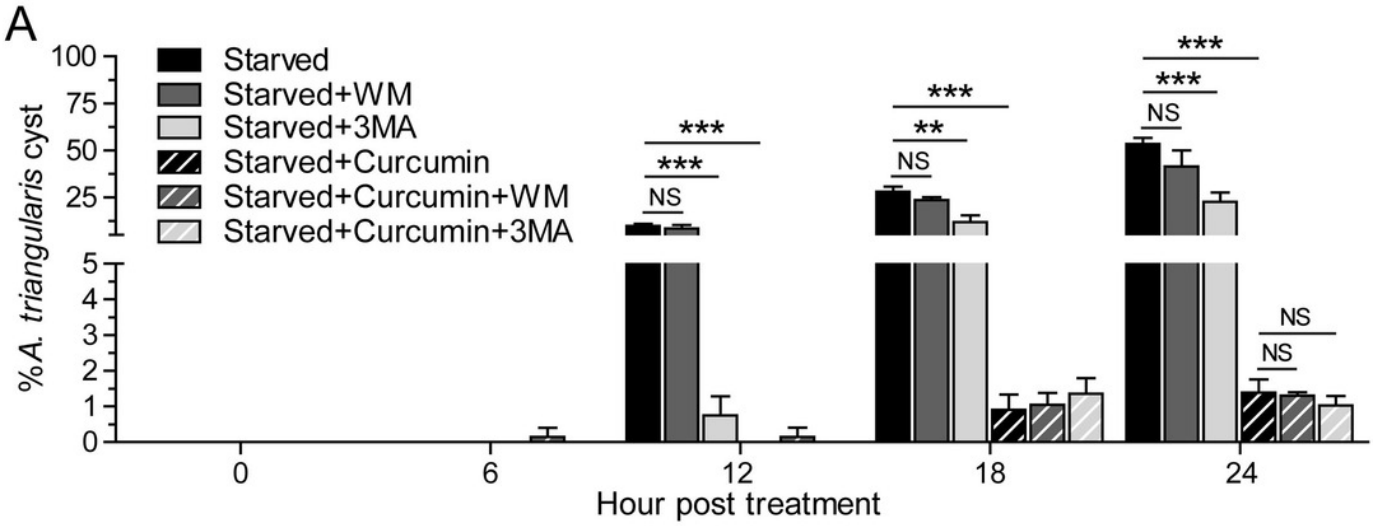
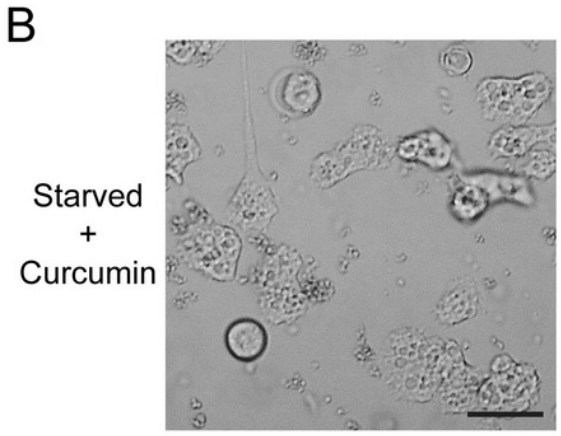

B
Full
Starved

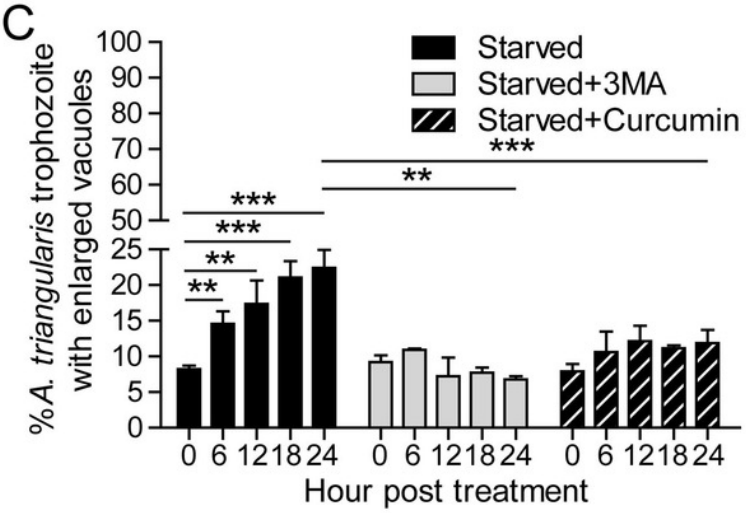
Figure 2

A. triangularis response to curcumin under starved condition.

(A) Cysts formation, the trophozoites were cultured in a starvation medium, PAS+5% glucose, with autophagy inhibitors or 50 µg/mL curcumin ± autophagy inhibitors for 24 h. Starvation alone was included as a positive control. Cyst was quantified every 6 h post-treatment. The percentage of cyst was calculated and represented as mean±SD. Data was obtained from 3 independent experiments. NS, not significant; **, $P<0.01$; ***, $P<0.001$. **(B)** Representative image of curcumin-treated parasites under starved condition. Bar 20 µM. **(C)** Vacuolization in surviving trophozoites, at least 100 cells, the trophozoites per condition were examined for enlarged vacuole, a diameter of at least 5 µm. Data was obtained from 3 independent experiments and represented as a mean percentage±SD. **, $P<0.01$; ***, $P<0.001$. **(D)** Transcriptional expression of autophagy-related genes after curcumin treatment, *A. triangularis* trophozoites were cultured in starvation medium with or without 50 µg/mL curcumin for 24 h. The parasites were harvested every 6 h, and the mRNA level of ATG3, ATG8b, ATG12, ATG16 genes were analyzed by qPCR. Their expression at each time point was expressed as a relative mRNA expression. The 18S rRNA was included as a reference gene. The expression at time 0 h was set to 1. The data were obtained from 3 independent experiments. Bar graphs represent mean±SEM.


A
Starved
Starved+WM
Starved+3MA
Starved+Curcumin
Starved+Curcumin+WM
Starved+Curcumin+3MA
%A. triangularis cyst
Hour post treatment
NS
***
**


B
Starved
+
Curcumin


C
Starved
Starved+3MA
Starved+Curcumin
%A. triangularis trophozoite with enlarged vacuoles
Hour post treatment

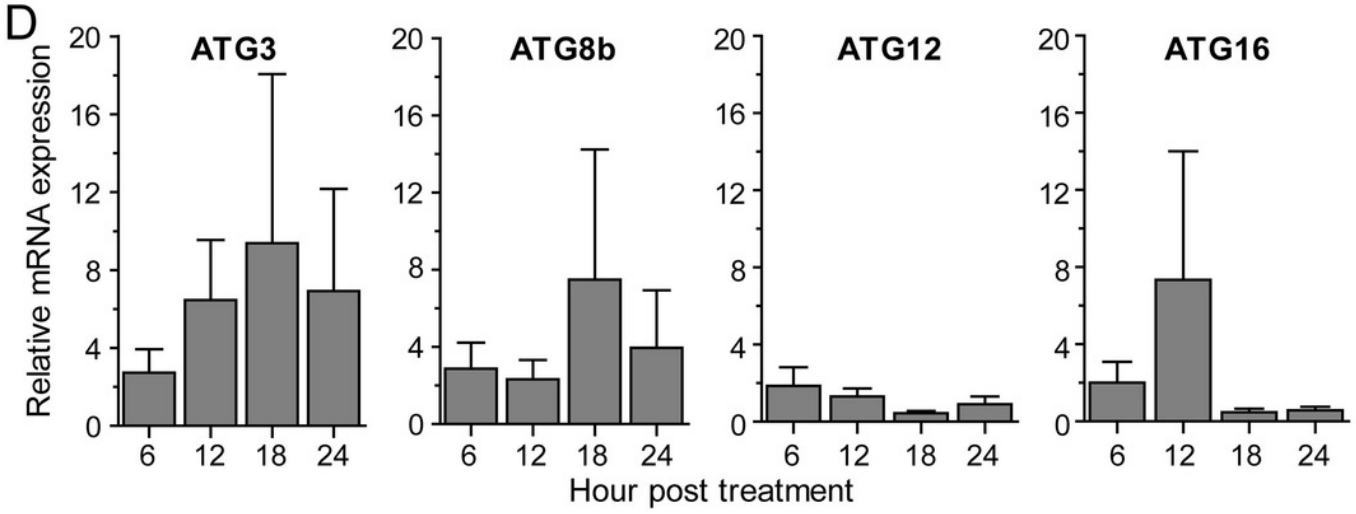

D
ATG3
ATG8b
ATG12
ATG16
Relative mRNA expression
Hour post treatment

Figure 3

A. triangularis response to curcumin under a nutrient-rich condition.

(A) Cyst formation, the *Acanthamoeba* trophozoites were cultured in a PYG medium with or without 50 µg/mL curcumin for 24 h. Starvation was included as a positive control for cysts formation. Cysts were quantified every 6 h post treatment. A percentage of cysts was calculated and represented as mean±SD. Data obtained from 3 independent experiments. NS, not significant; ***, $P<0.001$. **(B)** Representative image of curcumin-treated parasites under a full condition. Bar 20µm. **(C)** Vacuolization in surviving trophozoites, at least 100 cells the trophozoites per condition were examined for enlarged vacuoles, a diameter of at least 5 µm. Data obtained from 3 independent experiments and represented as a mean percentage±SD. NS, not significant. **(D)** Transcriptional expression of autophagy-related genes after curcumin treatment, *A. triangularis* trophozoites were cultured in PYG medium with or without 50 µg/mL curcumin for 24 h. The parasites were harvested every 6 h and the mRNA level of ATG3, ATG8b, ATG12, ATG16 genes were analyzed by qPCR. Their expression at each time point was expressed as a relative mRNA expression. 18S rRNA was included as a reference gene. The expression at time 0 h was set to 1. The data were obtained from 3 independent experiments. Bar graphs represent mean±SEM.

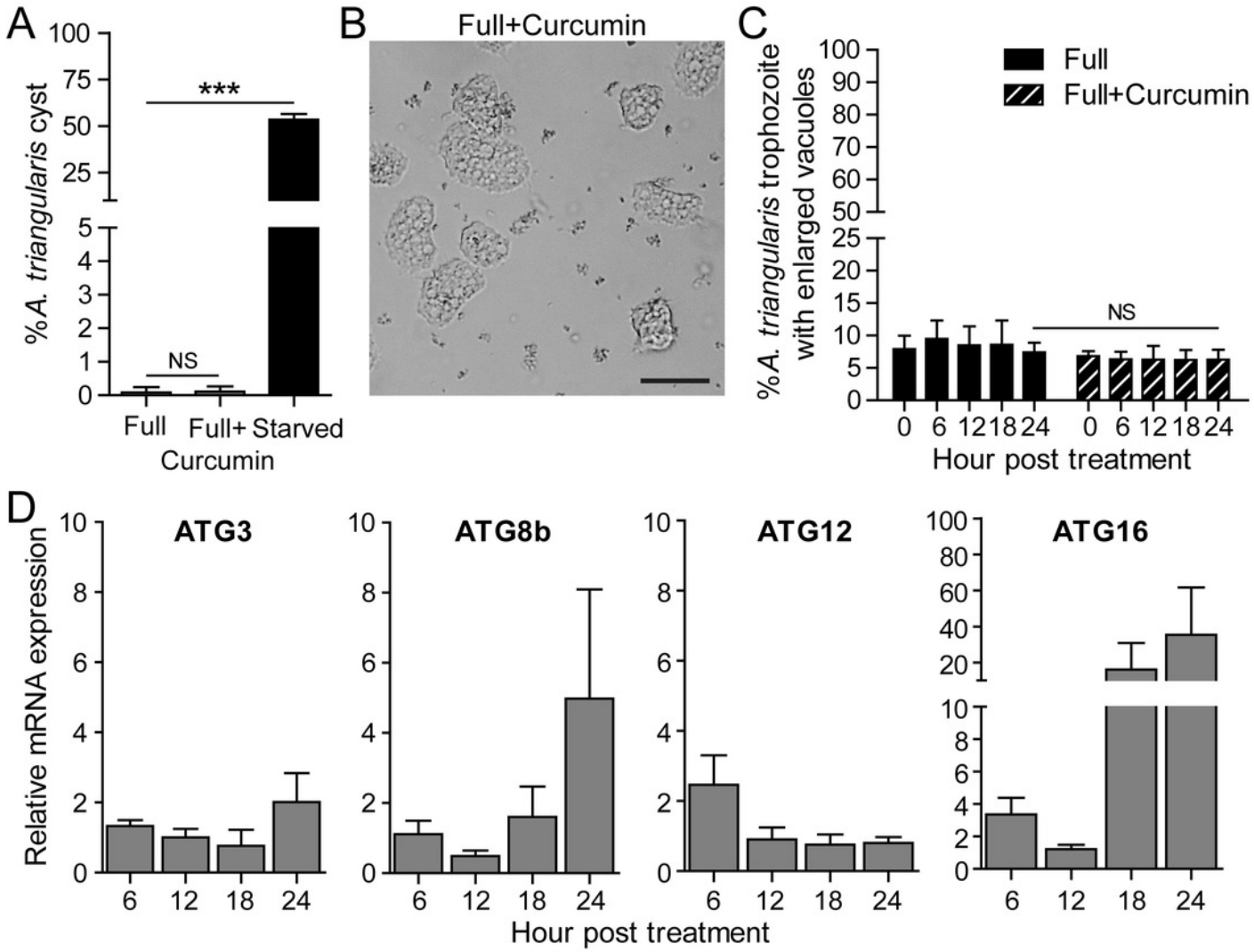

A
%A. triangularis cyst
***
NS
Full
Full+ Curcumin
Starved
B
Full+Curcumin
C
%A. triangularis trophozoite with enlarged vacuoles
Full
Full+Curcumin
NS
Hour post treatment
D
Relative mRNA expression
ATG3
ATG8b
ATG12
ATG16
Hour post treatment

Figure 4

Transcriptional expression of other encystation-related genes under a nutrient-rich condition.

Investigation of cellulose synthase (CS) and serine proteinase (SP) mRNA expression was carried out. cDNA samples were shared with autophagy analysis. The qPCR was performed, and 18S rRNA was included as a reference gene. The data were obtained from 3 independent experiments. Bar graphs displayed mean±SEM. **, $P<0.01$; ***, $P<0.001$.

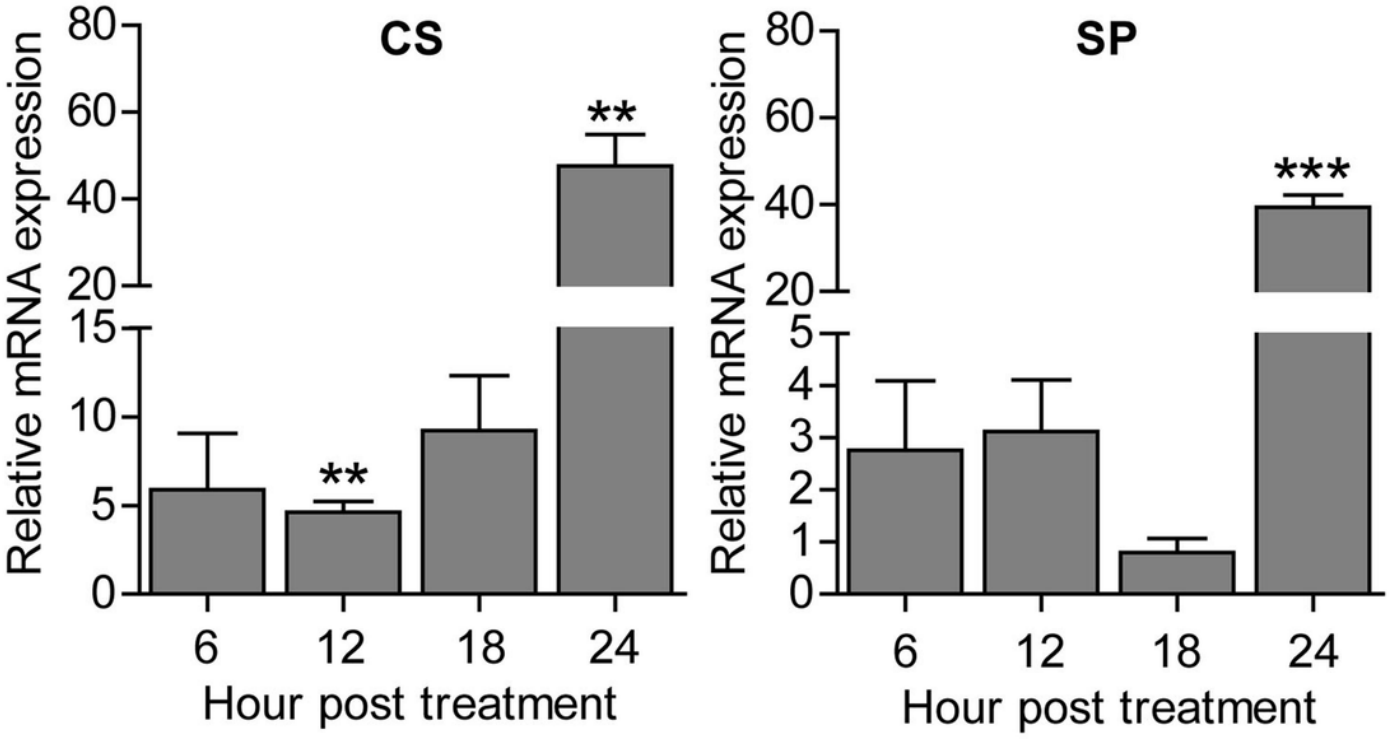
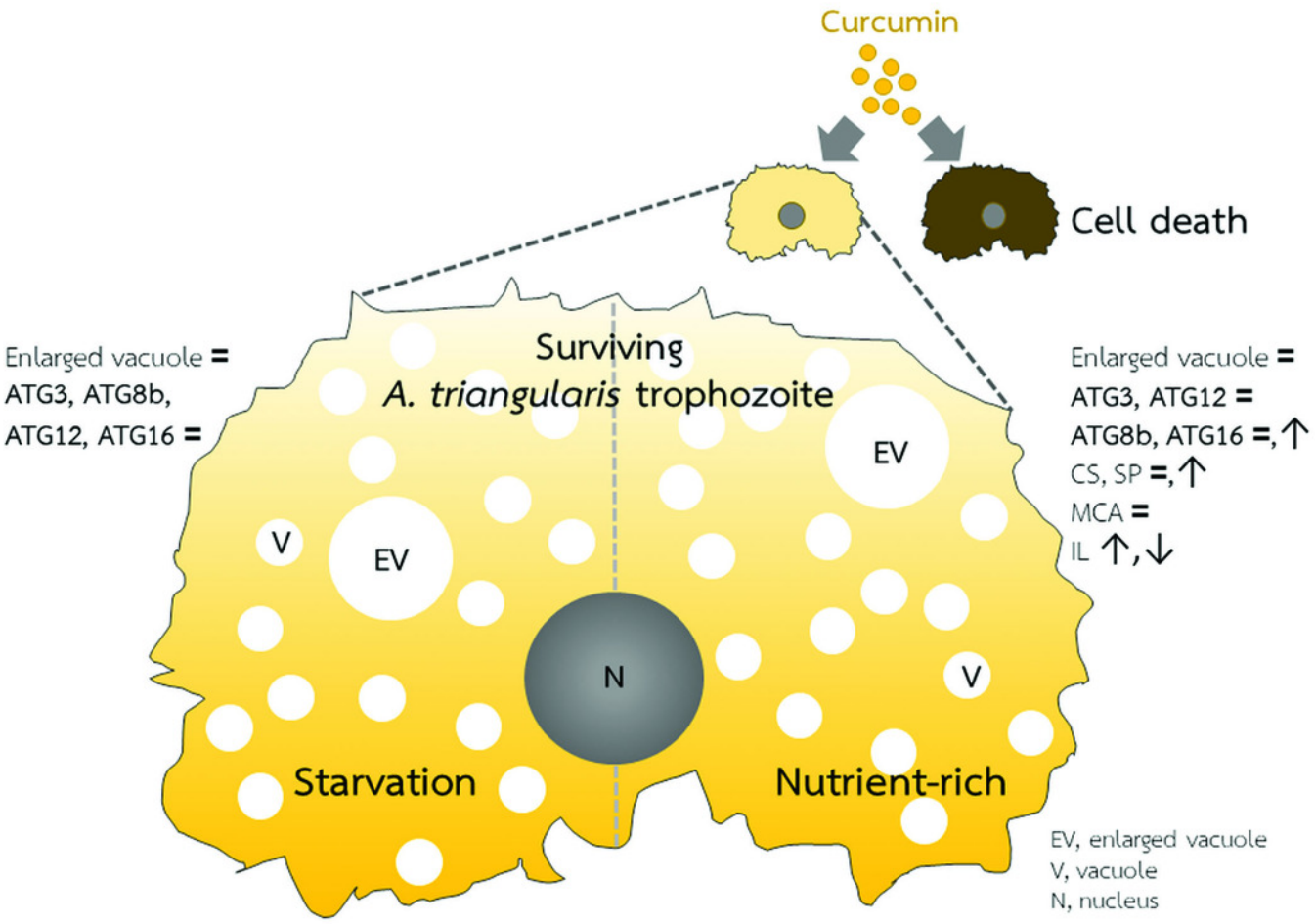


Figure 5

A. triangularis response to curcumin.

Curcumin at 50 µg/mL was used for *A. triangularis* trophozoites treatment. Approximately 50% of the parasites died, while the surviving parasites remained in the trophozoites stage. Transcriptional expression of tested autophagy-related genes was at the basal level in both starvation and a nutrient-rich condition except *Ac*ATG16, which increased at later time points under the nutrient-rich condition. Other *A. triangularis* encystation-related genes tested in this study, cellulose synthase and serine proteinase, were also increased at a later time point. Inducing an arrest in the trophozoites by curcumin is possibly resulting in the deactivation of the ATG genes and subsequent inhibition of vacuoles maturation.


Curcumin
Cell death
Enlarged vacuole =
ATG3, ATG8b,
ATG12, ATG16 =
Surviving
A. triangularis trophozoite
Enlarged vacuole =
ATG3, ATG12 =
ATG8b, ATG16 =, ↑
CS, SP =, ↑
MCA =
IL ↑, ↓
EV
V
EV
N
V
Starvation
Nutrient-rich
EV, enlarged vacuole
V, vacuole
N, nucleus