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Nordihydroguaiaretic acid (NDGA) and α-mangostin inhibit the growth of *Mycobacterium tuberculosis* by inducing autophagy

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ABSTRACT

Tuberculosis (TB) remains as a global health problem. The prevalence of this infection is related to the association with other diseases, such as HIV, neglect treatment and misuse of antibiotics. Hence, the identification of new drugs is required to eradicate TB. Possible alternatives to existing antibiotics include pure compounds extracted from medicinal plants, which are an important source of antimicrobial agents. The aim of this study was to evaluate the effect of nordihydroguaiaretic acid (NDGA) and α-mangostin on *Mycobacterium tuberculosis* growth and bacterial survival in infected macrophages derived from the human THP-1 cell line and monocytes. Our results show that both compounds directly inhibit *M. tuberculosis* growth in liquid medium with Minimal Inhibitory Concentrations (MIC) of 250 and 62 μg/mL respectively, likely through preventing bacterial replication. In addition, NDGA and α-mangostin were able to induce autophagy in human cells at lower concentrations (7 and 6 μg/mL, respectively) and contributed to the elimination of intracellular bacteria. NDGA and α-mangostin could be candidates for coadjuvant therapy in cases of drug-resistant TB, and their ability to enhance the immune response by promoting autophagy might contribute to TB treatment.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and remains a global health problem. It is estimated that 9.0 million people developed this infection and 1.5 million died from TB in 2013 (WHO, 2014). The prevalence of TB is related to neglect treatment and the misuse of drugs as well as patient complications from other diseases, such as diabetes mellitus and HIV [1].

In the last decade, there has been a renewed of focus on the development of drugs to treat TB, and several compounds are evaluated in clinical trials (WHO, 2013). However, despite the small but growing number of drugs that are effective at killing *M. tuberculosis*, the current treatment is still burdened by its duration (typically 6 months for drug-sensitive strains) and the ever-increasing number of multidrug-resistant (MDR) and extensively drug-resistant (XDR) clinical isolates of *M. tuberculosis* [2].

Antibiotic resistance now represents a devastating problem for human health because pathogens have developed mechanisms to evade all available antibiotics [3]. Thus, the development of new antimicrobial agents and strategies for the treatment of TB are fundamental for its eradication.

The existing drugs used for treating TB primarily target metabolic reactions and proteins that are essential for the active growth of *M. tuberculosis* [4]. Currently, the search is focused on finding new drugs that can act on molecular targets related to the establishment of mycobacterial dormancy in human macrophages or inhibit bacterial virulence factors that interfere with the signaling pathways of host cells and affect their immunocompetence, leading to the persistence of the disease [5].

One way to develop new drugs is by studying the biological activity of natural products, semi-synthesis or the generation of new compounds [6–9]. The great diversity of natural compounds represents a powerful tool for drug discovery; such compounds could be applied, as new drugs with potential antimicrobial activity themselves or by a modulating the immune response to enhance the removal of the infective agent [10].

Larrea tridentata and *Garcinia mangostana* are widely used to treat diverse diseases, including infectious diseases such as influenza, flu and even TB [11–14]. It has been experimentally demonstrated that extracts from *L. tridentata* and *G. mangostana* show bactericidal activity against strains of *M. tuberculosis* [13,15,16].

Research on *L. tridentata* has shown that this species is a remarkable source of bioactive compounds. The resin covering its leaves contains 19 flavonoid aglycones as well as several lignans, including NDGA [17]. A

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number of glycosylated flavonoids, sapogenins, essential oils, waxes and alkaloids have been isolated from the bark and roots. Among the most abundant compounds found in *L. tridentata* is NDGA. This compound accounts for between 5 and 10% of the total dry weight of the leaves and is the most abundant phenol resin (>80%) [11,17]. Extensive studies have shown that NDGA presents beneficial properties, including antioxidant, antitumor, antiviral, antibacterial and anti-inflammatory activity [12,18].

NDGA has been demonstrated to selectively inhibit arachidonic acid 5-lipoxygenase activity, which in turn reduces leukotriene and prostaglandin synthesis, thus leading to a reduction of inflammatory activity. NDGA also has profound effects on the secretory pathway, reflected in its ability to block the production of leukotriene B4, degranulation, phagocytosis, and the respiratory burst [19]. Furthermore, NDGA reduces cytokine production, including that of IL-4, IL-5, IL-13, and TNF-α in human cell lines and murine models [20].

In addition, the pericarp of *G. mangostana* has a wide diversity of compounds as xanthones, benzophenones, bioflavonoids and triterpenes [21–23]. The α-mangostin is the most abundant xanthone of *G. mangostana*; it yields 30–50% from pericarp [16,24]. Several studies have shown that α-mangostin has remarkable biological activities as antioxidant, antitumoral, anti-inflammatory, antiallergy, antibacterial, antifungal and antiviral [14]. Recently, it was demonstrated that α-mangostin selectively induced autophagy in chronic myeloid leukemia (CML) cell lines and human glioblastoma cells [25,26]. α-Mangostin increases the expression level of the autophagosome markers Beclin1 and LC-3II, and promote the accumulation of autophagic vesicles [26].

In this study, the objective was to evaluate the effect of NDGA and α-mangostin on the survival of *M. tuberculosis H37Ra* in vitro and the intracellular survival of bacteria in infected THP-1 cells and in human monocyte derived macrophages (MDMs).

2. Materials and methods

2.1. Reagents

NDGA or α-mangostin was dissolved in dimethyl sulfoxide (DMSO), all of these were purchased from Sigma-Aldrich (St. Louis, MO, USA) to a concentration of 20 mg/mL and then further diluted in culture media. Autophagy inhibitors SB 203580 (SB) were acquired from Promega (Madison, WI, USA) and Wortmannin from Calbiochem (USA). NDGA and α-mangostin were lipopolysaccharide (LPS) free as determined by Limulus Amebocyte Lysate Pyrogent 03 assay kit (Lonza, Walkersville, MD, USA).

2.2. Bacterial culture conditions

M. tuberculosis H37Ra was purchased from the American Type Culture Collection (ATCC 25177, Rockville, MD, USA). It was routinely grown at 37 °C in 7H9 broth (Difco, Detroit, MI, USA) supplemented with ADC (Beckton Dickinson, BD, San Jose, CA, USA) and 0.2% glycerol or on solid Middlebrook 7H10 medium (Difco) supplemented with 0.5% glycerol and OADC (BD). We also used a strain of *M. tuberculosis* H37Ra that contains the pCherry8 plasmid (Plasmid number 24663, provided by Addgene, Cambridge, MA, USA). This plasmid expresses the fluorescent reporter protein mCherry from the P_{smyc} promoter [27]. *M. tuberculosis* H37Ra was transformed with the pCherry8 plasmid via electroporation [28]. Then, the bacteria were grown in 7H9 Middlebrook medium supplemented with OADC, 0.05% Tween-80 (Sigma-Aldrich), and 50 μg/mL hygromycin B (Roche Life Science, Indianapolis, IN, USA). Prior to infection, *M. tuberculosis* was disaggregated to achieve a single-cell suspension. For this purpose, a bacterial stock was thawed and centrifuged at 3000 ×*g* for 8 min and washed in RPMI medium without antibiotics. The bacterial suspension was subsequently passed through a 21 G needle (10 times) and then a 1 G needle (10 times), followed by sonication for 1 min. Residual clumps were removed via centrifugation at 500 ×*g* for 1 min.

2.3. THP-1 cell line and MDMs

The human acute monocytic leukemia cell line THP-1 was purchased from the American Type Culture Collection (TIB202, ATCC, Rockville, MD, USA). THP-1 cells were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate (Lonza), 10% heat-inactivated fetal bovine serum (HycloneTM, Logan, USA) and 50 μM β-2-mercaptoethanol (Bio-Rad Laboratories, Berkeley, CA, USA). Cultures were continuously maintained at a cell density between 0.2×10^6 and 1×10^6 cells/mL. To induce differentiation of cells to macrophages, THP-1 cells (0.15×10^6 cells/well) were cultured on 96-well plates using 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). After 3 days, medium was removed, and fresh medium was immediately added. The cells were incubated in control medium by 4 h before any treatment [29].

MDMs were obtained from peripheral blood mononuclear cells (PBMCs) through the centrifugation of buffy coats from healthy blood bank donors at the Instituto Nacional de Enfermedades Respiratorias (INER) under approbation of the Institutional Ethical Review Board of INER. Heparinized blood was diluted 1:1 with RPMI and centrifuged at 300 ×*g* for 45 min at room temperature over lymphocyte separation solution (Lonza) [30]. The layer containing PBMCs was harvested and monocytes were enriched by plastic adherence. PBMCs were plated on 100 mm Petri dishes (Costar, NY USA) and incubated for 1 h at 37 °C, in 5% CO2. After discarding non-adherent cells and three washings, the monocytes (MN) were recovered. The viability of MN as assessed by Trypan blue exclusion was higher than 98%. MN concentrations were adjusted to 1×10^6 cells/mL and cultivated in RPMI 1640 supplemented with 200 mM L-glutamine, and 10% heat-inactivated human serum 24-well plates (1×10^6 cells/well) at 37 °C, in 5% CO_2 for 7 days. By this time, the cells had acquired macrophage morphology [31,32].

2.4. THP-1 cell viability

The cells were seeded at a density 0.15×10^6 cells/well in 96-well plates in culture medium and differentiated with PMA over three days. The cells were either infected or left as uninfected controls, and NDGA or α-mangostin were immediately added to the cells. The cells were then incubated for five days and to avoid exhaustion of stimuli in medium, it was replaced twice during incubation period at days 2 and 4 with media containing freshly prepared stimuli. After incubation, cell viability was quantified using a colorimetric assay based on metabolic activity (Cell Titer 96 Aqueous One Solution distributed by Promega, Madison, WI, USA), according to the manufacturer's instructions.

2.5. Evaluation of NDGA and α-mangostin antimycobacterial activity in bacterial cultures

For these assays, NDGA or α-mangostin endotoxin free were added at concentrations ranging from 1000 to 1.0 μg/mL, and each well was inoculated with a bacterial suspension at a concentration of 10^5 colony-forming units (CFUs)/mL for total volume of 200 μL. The plates were incubated for 7 days at 37 °C. Subsequently, the CFUs were counted as previously described [33], and the minimum inhibitory concentration (MIC) was determined. No inhibitory effects were observed in the presence of DMSO at the concentration used in these assays (<1.25%). Rifampicin at 0.4 μg/mL (Sigma-Aldrich) was employed as a reference bactericidal drug for *M. tuberculosis.*

2.6. Evaluation of effect of NDGA and α-mangostin on mycobacterial growth

The infection of differenced THP-1 cells (0.15×10^6/well on the 96-well plate) and MDMs (1.0×10^6/well on the 24-well plate) were performed using bovine serum-opsonized *M. tuberculosis* at a multiplicity of infection (MOI) of 1:10 in RPMI medium without antibiotics. After 1.5 h of incubation at 37 °C in a 5% CO_2 atmosphere, the cells were washed to remove non-internalized bacteria. Immediately after, cells were stimulated with freshly prepared NDGA, α-mangostin, or rifampicin diluted in RPMI medium containing 10% of heat-inactivated human serum at declared concentrations, and incubated at 37 °C in 5% CO_2 for five days. To avoid exhaustion of stimuli in medium, it was replaced twice during incubation period at days 2 and 4. After the indicated period of time, the intracellular bacteria were quantified. The cells were disrupted with 0.1% SDS for 10 min and then neutralized via the addition of 10% BSA. Then, the bacteria were quantified by CFUs counting or *M. tuberculosis* viability was assessed by real time PCR-based method [34], which compares 16S RNA levels with genomic DNA (IS6110) levels as an indicator of bacterial growth. Total RNA was isolated from cell lysates by disruption with small glass beads [35] in the presence of TRIzol reagent (Life Technologies, CA, USA) followed by RNA cleanup using an RNeasy Miniprep Kit (QIAGEN, CA, USA). cDNA was synthesized using the Script cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The relative 16S values were calculated using ΔΔCT analysis, with the IS6110 value serving as the "housekeeping gene". The following IS6110 genomic element and 16S primer sequences were used: 16S Forward 5′-GGT GCGAGCGTTGTCCGGAA-3′, 16S Reverse 5′-CGCCCGCACGCTCACAGTTA-3′ and IS6110 Reverse 5′-TCTTGTATAG GCCGTTGATCGTCT-3′ [34].

2.7. Autophagy inhibition

Additionally, we evaluated the effect of autophagy inhibitors (SB and wortmannin) on survival of *M. tuberculosis* in THP-1 cells and MDMs. After the time of infection and removed non-internalized bacteria, the cells were incubated with SB (10 μM) or wortmannin (100 nM) by 0.5 h and then NDGA was added. Finally, the cells were incubated for 3 days, lysed and the intracellular bacteria were quantified by CFUs and, real time PCR-based method.

2.8. LC3 immunofluorescent staining

For microscopy experiments, 1×10^5 cells/well in 500 μL of RPMI medium were cultivated in an 8-well Lab-Tak II chamber. After the cells were infected with *M. tuberculosis* expressing the mCherry fluorescent protein and subjected to NDGA or α-mangostin treatment, they were stimulated as described above. Next, the cells were fixed with 4% paraformaldehyde, stained with rabbit anti-LC3B (Enzo Life Sciences, NY, USA) and visualized with Fluorescein Isothiocyanate (FITC). Counter-staining with Hoechst (Enzo Life Sciences) to detect nuclei was performed following the manufacturer's instructions. Cells were visualized under a fluorescence AxioScope.A1 microscope (Carl Zeiss, Oberkochen, Germany), and images were acquired and analyzed with ZEN Pro software (Carl Zeiss) [36].

2.9. Measurement of TNF-α

Infected THP-1 cells and MDMs were treated with NDGA and autophagy inhibitors (wortmannin or SB). Culture medium alone was used as a negative control and supernatants of treated cells were harvested and frozen at −20 °C. TNF-α production was determined in 24 h culture supernatants using an enzyme-linked immunosorbent assay (ELISA) previously described [37]. Absorbance was read on an HT Multi-Mode Microplate Reader (Biotek, WA, USA) at 450 nm. The results are presented as the mean value for duplicate wells in three independent experiments.

2.10. Statistical analyses

Statistical analyses were performed with GraphPad Prism through analysis of variance (ANOVA) with Dunnett's multiple comparison tests for THP-1 cell line and Friedman's multiple comparison tests for MDMs. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. NDGA and α-mangostin show inhibitory activity against the growth of *M. tuberculosis*

First, we evaluated the effect of NDGA on *M. tuberculosis* growth and determined that this compound caused a dose-dependent decrease in bacterial growth at doses from 8 to 250 μg/mL when the bacteria were cultured in liquid medium (Fig. 1A). The bactericidal effect of NDGA was compared with those of rifampicin (0.4 μg/mL) and α-mangostin,

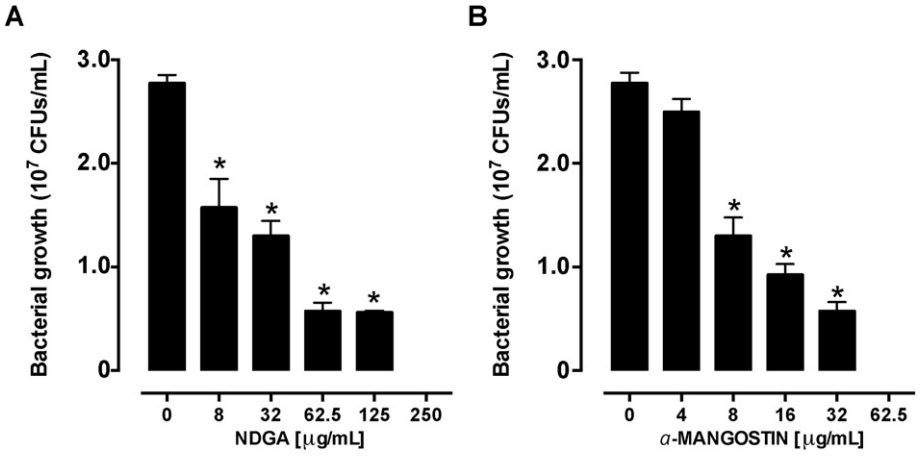

Fig. 1. Inhibitory effects of NDGA and α-mangostin on *M. tuberculosis* growth. Bacteria were cultivated in 7H9 Middlebrook medium supplemented with NDGA (A) or α-mangostin (B) for 7 days. CFUs were quantified by counting the number of surviving bacteria for each concentration of compounds. The presented data represent the mean ± SE ($n = 4$). *$p < 0.05$ compared to cells treated with media alone.

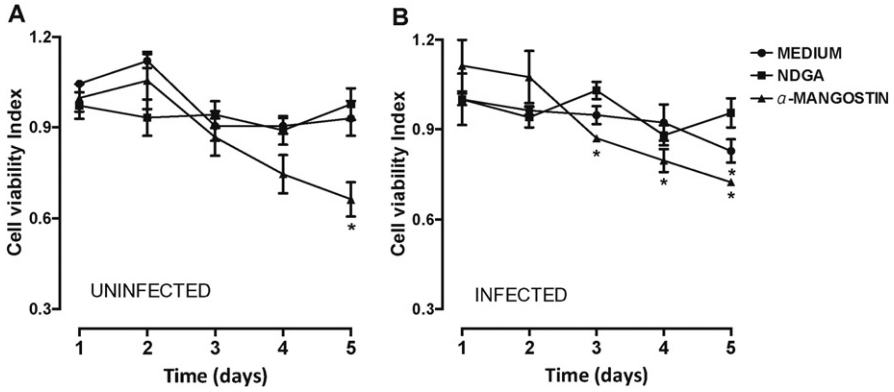

Fig. 2. Effect of NDGA (7 μg/mL) and α-mangostin (6 μg/mL) on the viability of THP-1 macrophages that were left uninfected (A) or infected with *M. tuberculosis* H37 Ra (B). Data represent the mean ± SE (n = 4). *p < 0.05 compared to the untreated cells.

a natural compound with previously described antimycobacterial activity (Fig. 1B) [38]. The obtained MICs were 250 μg/mL and 62.5 μg/mL for NDGA and α-mangostin, respectively.

3.2. Effect of NDGA and α-mangostin on THP-1 cell viability

NDGA did not significantly decrease THP-1 cell viability in either infected or uninfected cells after 5 days of incubation; however, α-mangostin promoted cell death under both conditions. The viability of the infected cells was decreased significantly after 3 and 5 days of incubation, while the viability of uninfected cells was reduced after 5 days of incubation. In addition, the infected cells that received no treatment (medium) showed a slight decrease in viability after 5 days of incubation (Fig. 2A-B).

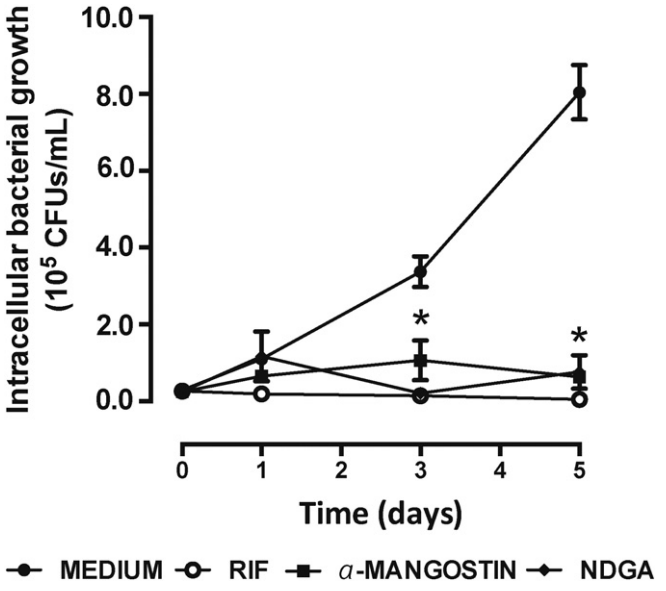

Fig. 3. Intracellular antimicrobial activity of NDGA and α-mangostin against *M. tuberculosis* in THP-1 macrophages. NDGA (7 μg/mL) and α-mangostin (6 μg/mL) were added 1 h after infection. CFUs were quantified by counting the numbers of surviving bacteria at each time point. The presented data represent the mean ± SE (n = 4). *p < 0.05, significant difference of each treatment (RIF, NDGA or α-mangostin) with respect to cells treated with medium only.

3.3. NDGA and α-mangostin inhibit the intracellular growth of *M. tuberculosis* in THP-1 cells

We found that post-infection treatment with NDGA or α-mangostin endotoxin free decreased the mycobacterial burden in THP-1 cells in comparison with untreated cells after 3 and 5 days of incubation (Fig. 3). However, neither NDGA nor α-mangostin were capable of completely inhibit the growth of bacteria in a manner comparable to rifampicin. Intracellular bacterial growth was significantly decreased in THP-1 cells treated with NDGA or α-mangostin ($p < 0.05$, 75% and 50%, respectively) in comparison with medium after 5 days of incubation. Treatment with rifampicin resulted in a significant bactericidal effect of more than 98% throughout the incubation period.

3.4. NDGA and α-mangostin induce autophagy in THP-1 cells

During autophagocytosis, the LC3 protein migrates from the intracellular cytoplasm to autophagic vesicles; thus, concentrated vesicles containing fluorescent LC3 indicate autophagy [39]. NDGA (7 μg/mL) increased the percentage of cells showing LC3 expression (80%) with respect to the medium alone (26%) (Fig. 4A-C). Fluorescence microscopy revealed a visible increase in the number and size of intracellular autophagic vesicles in the cells treated with NDGA. The treated cells exhibited an average of 18 puncta, while the control only resulted in approximately 9 puncta per cell (Fig. 4C). In addition, we demonstrated that α-mangostin (6 μg/mL) induced autophagy in THP-1 cells. Under these conditions, 84% of the cells showed autophagosome formation, and the average number of puncta per cell was 20. We also evaluated the effect of post-treatment with NDGA and α-mangostin on autophagy in macrophages infected with *M. tuberculosis.* These cells showed significant increment in the number of vesicles and in the detection of bacteria within autophagic vesicles with respect to the control (70% and 78% of cells with vesicles, under treatment with NDGA and α-mangostin, respectively) (Fig. 5A-C).

3.5. Autophagy inhibition decreased antimicrobial activity of NDGA in macrophages infected with *M. tuberculosis*

To investigate the role of the interaction between antimicrobial activity and autophagy induced by NDGA. THP-1 cells and MDMs were infected, and then treated with SB or wortmannin for 0.5 h and immediately NDGA added and incubated for 24 h. As shown, the treated THP-1 cells with W or S plus NDGA significantly diminished LC-3II accumulation ($p < 0.05$, 30% and 22% respectively) compared to NDGA alone. We also evaluated the effect of the autophagy inhibitors on intracellular survival of *M. tuberculosis.* We observed that antimicrobial activity

induced by NDGA was significantly reduced in THP-1 treated with W or SB after 3 days of incubation (Fig. 6D). Similarly, we evaluated the effect of autophagy inhibitors in MDMs infected with *M. tuberculosis* and observed that antimicrobial activity induced by NDGA was decreased after treatment with wortmannin (Fig. 6E). In addition TNF-α levels were assessed in supernatants from infected THP-1 cells and MDMs treated with NDGA and with autophagy inhibitors (wortmannin and SB). We observed that the TNF-α production was significantly lower in cells treated with NDGA in comparison to medium and that autophagy inhibitors did not restore the effect caused by NDGA (Fig. 6C) in both cells THP-1 and MDMs.

4. Discussion

According to the WHO, more than 75% of the world's population employs plants as a form of health care [40,41]. In fact, there are 350 described species of plants used around the world for the treatment of TB, including *L. tridentata* [8,15]. It is noteworthy that a number of compounds with distinct biological activities have been isolated from

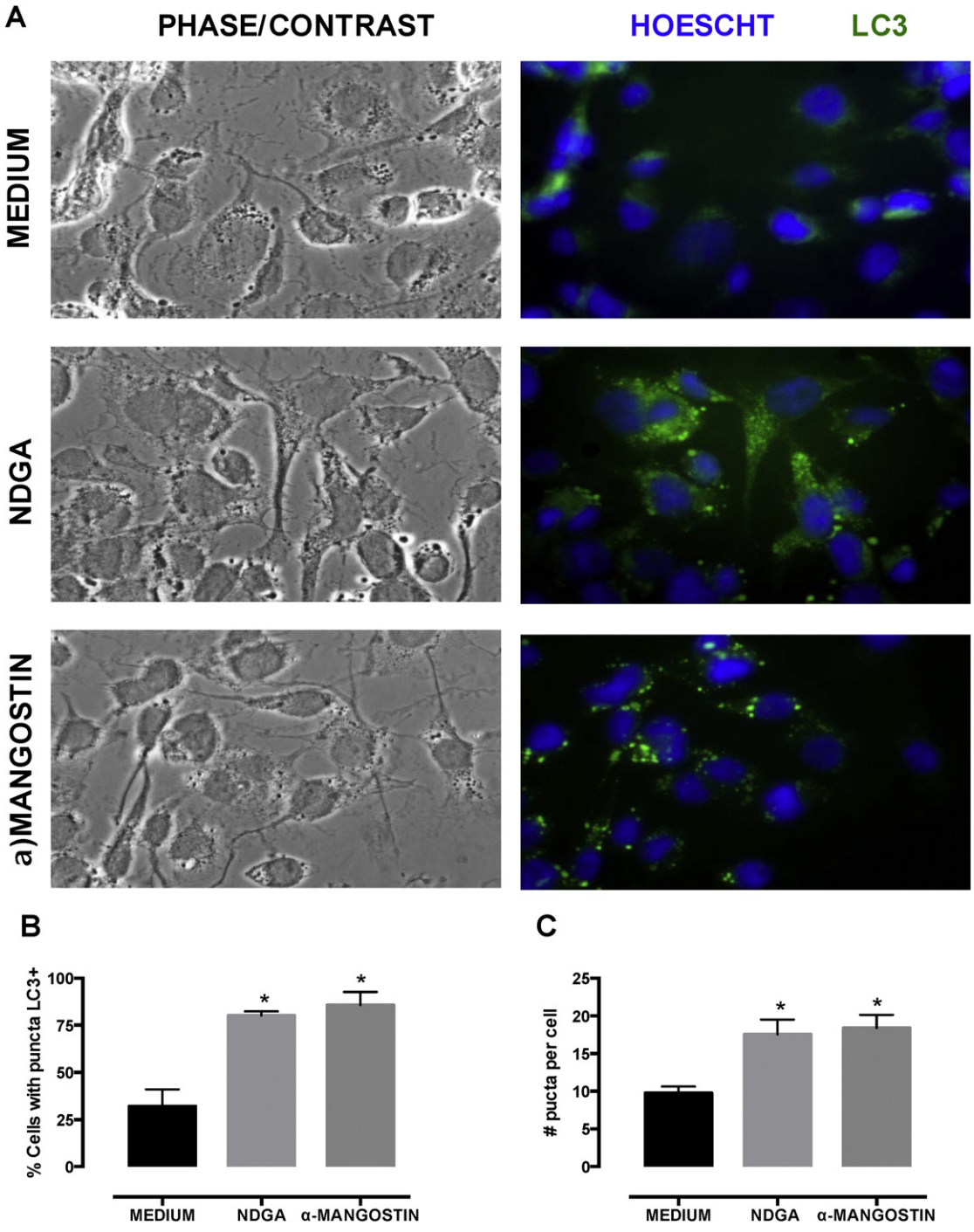

Fig. 4. NDGA and α-mangostin induced autophagy. Cells were stimulated with 7 μg/mL NDGA or 6 μg/mL α-mangostin for 24 h. (A) Detection of autophagosomes was performed via fluorescence microscopy using an anti-human LC3-FITC antibody; images acquired at 1000× are shown. (B). The percentage of cells with LC3+ puncta was assessed by counting a minimum of 80 cells for each condition. (C) The number of puncta per cell was counted in at least 25 of the cells showing puncta. *$p < 0.05$, significant difference of each treatment (NDGA or α-mangostin) with respect to cells treated with medium only.

medicinal plants [42]. In this work, we evaluated the effects of NDGA on antimycobacterial activity and cytotoxicity in infected or uninfected THP-1 cells. First, based on the results obtained using the CFU counting, we demonstrated that NDGA had an inhibitory effect on *M. tuberculosis*. The results showed that NDGA partially decreased bacterial growth in vitro at concentrations ranging from 8 to 125 μg/mL, and exerted a complete bactericidal effect on *M. tuberculosis* at 250 μg/mL. This activity may be related to the structural similarity of this compound with flavonoids, as there is evidence that some flavonoids, such as butein and isoliquiritigenin, exhibit complex structures that destabilize the bacterial cell wall and inhibit the synthesis of fatty acids and mycolic acids [43,44]. Additionally, it has been reported that some phenols, such as meso-dihydroguaiaretic acid, that are similar to NDGA can inhibit the enzyme carbonic anhydrase, which is involved in carbon metabolism, and the coenzyme A transferase of *M. tuberculosis* [45,46], potentially preventing bacterial growth in vitro.

It has been reported that several xanthones isolated from the fruit hull of *G. mangostana*, such as α- and β-mangostins and garcinone B, exert an inhibitory effect against *M. tuberculosis* [13]. In our assay, we demonstrated that α-mangostin decreased mycobacterial growth at a concentration of 25 μg/mL. It was previously shown that α-mangostin quickly disrupted the bacterial wall of *Staphylococcus aureus*, leading to the loss of intracellular components, and it is possible that this compound interacts directly with the bacterial membrane, provoking membrane disruption and bactericidal action [47]. In addition, it has been observed that α-mangostin can inhibit enzymes involved in glycolysis, such as aldolase, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase, in *Streptococcus mutans* [48]. In this context, α-mangostin could dilute lipophilic substances such as wall

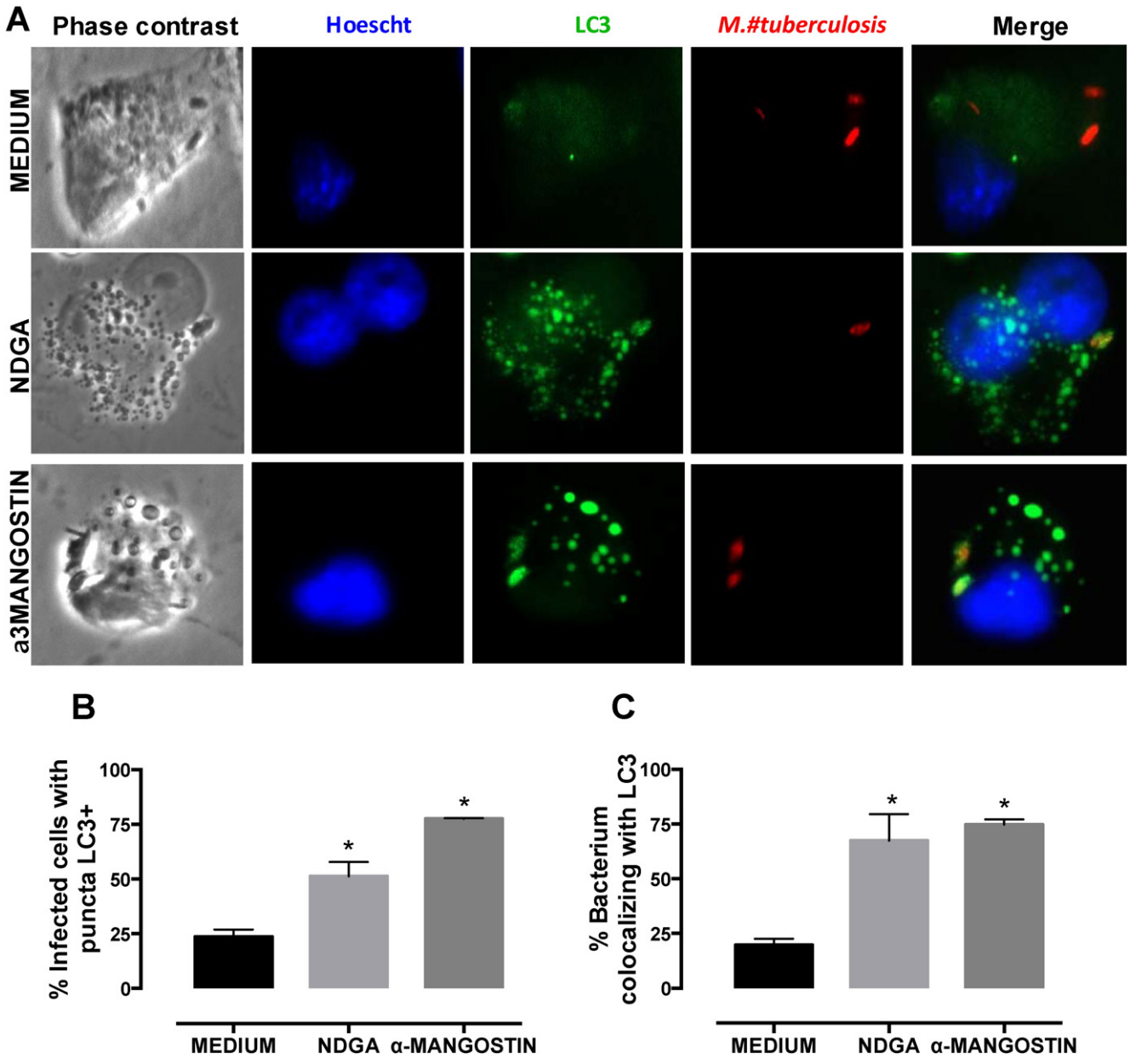

Fig. 5. NDGA and α-mangostin induced autophagy in infected THP-1 macrophages and *M. tuberculosis* is localized in autophagosomes. Cells were infected with *M. tuberculosis* and then stimulated with 7 μg/mL NDGA or 6 μg/mL α-mangostin for 24 h. (A) Detection of autophagosomes was performed via fluorescence microscopy using an anti-human LC3-FITC antibody. *M. tuberculosis* detection was conducted based on the expression of the mCherry fluorescent protein, and images were acquired at 1000×. (B) The percentage of cells with LC3+ puncta was assessed by counting a minimum of 80 cells in each condition. (C) Percentage of bacteria colocalizing with LC3 are shown. The number of puncta per cell was counted in at least 25 of the cells showing puncta. *$p < 0.05$, significant difference of each treatment (NDGA or α-mangostin) with respect to cells treated with medium only.

components and simultaneously inhibit a number of enzymes in *M. tuberculosis*, thus preventing bacterial growth.

We found that 7 μg/mL NDGA and 6 μg/mL α-mangostin significantly decreased the intracellular growth of *M. tuberculosis*. These results suggest that these two compounds could have inhibitory effects through two different mechanisms. High concentrations of NDGA or α-mangostin directly prevented the growth of *M. tuberculosis*, and the possible mechanisms were discussed above. However, lower concentrations of these compounds prevented the intracellular growth of the bacteria in macrophages, and these effects may have been due to stimulatory effects of NDGA or α-mangostin on the immune response in human macrophages.

Recently, autophagy has been identified as a mechanism to restrict mycobacterial proliferation in human macrophages [49]. Our results showed that α-mangostin induce autophagy in uninfected or infected THP-1 cells after 24 h of treatment and the autophagic vesicle colocalization with mycobacteria. However, after 3 days of incubation, treatment with α-mangostin resulted in the death of the infected cells, most likely through autophagy. It has been reported that α-mangostin can induce autophagy and autophagic cell death in mouse intestinal epithelial cells, GFP-LC3 transgenic mice [50], human glioblastoma cells [25] and CML cell lines [26].

To our knowledge, this is the first report of autophagy induction and antimycobacterial activity by NDGA. Our results also showed that treatment with the autophagy inhibitors wortmannin (PI3K inhibitor) and SB (p38 inhibitor), resulted in a marked increase in bacterial load and significant decrease of LC3-II puncta formation in THP-1 cells and MDMs. Therefore, our results suggest that NDGA induces autophagy

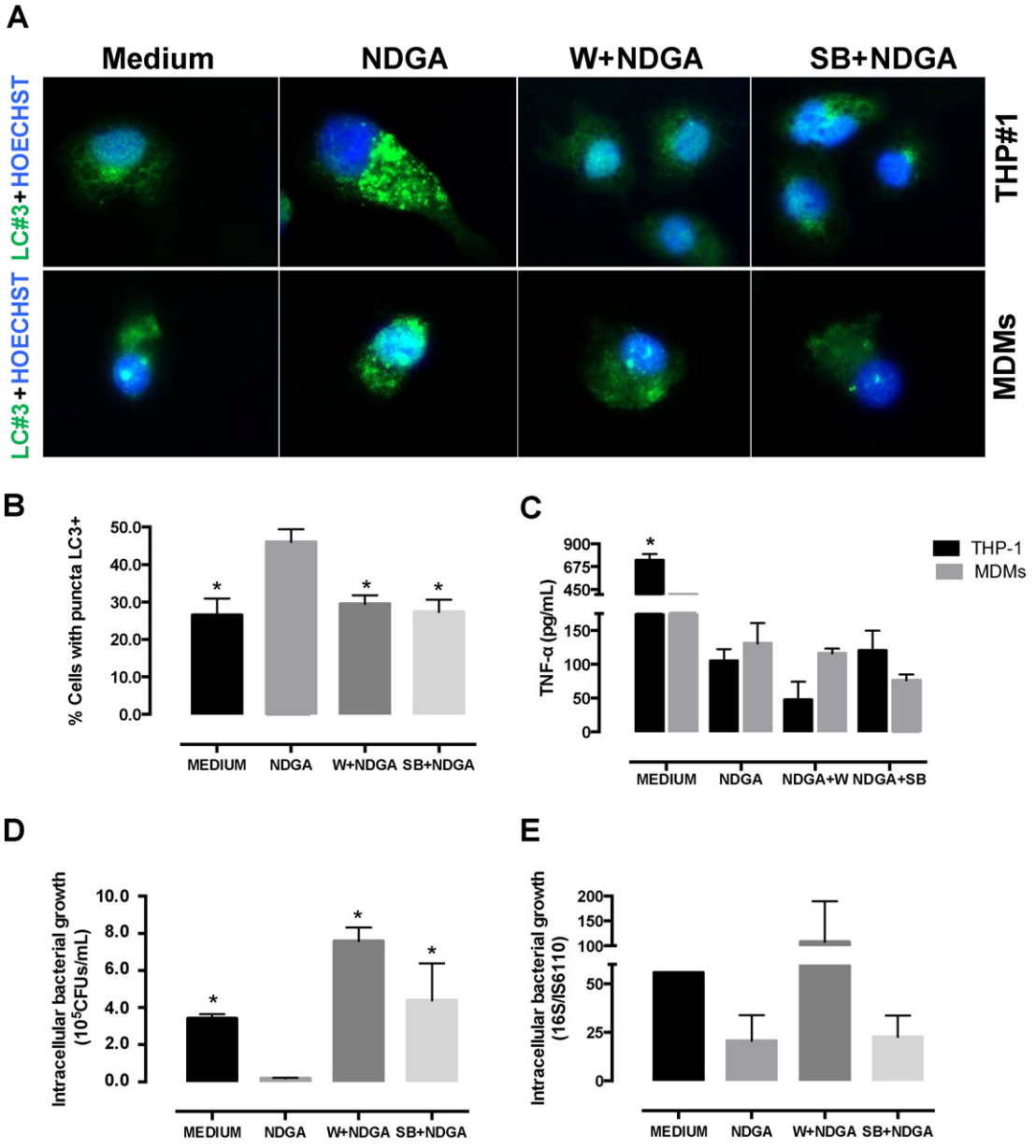

Fig. 6. Autophagy Inhibition decreased antimicrobial activity of NDGA in infected cells. THP-1 cells and MDMs were infected with *M. tuberculosis* at an MOI of 10 and incubated for 24 h. (W) wortmanin, and (SB) SB203580 were added and incubated for 0.5 h, then 7 μg/mL NDGA was added and incubated for additional 24 h. A) THP-1 cells (top) and MDMs (bottom) were fixed and stained with anti-LC3-FITC; Hoescht was used to stain the nuclei. Detection of autophagosomes was performed via fluorescence microscopy and images were acquired at 1000×. B) Graphics show the percentage of THP-1 cells with puncta LC3 upon the indicated treatments. C) TNF-α levels were assessed in 24 h in supernatants from infected THP-1 cells and MDMs. D) Mycobacterial growth was quantified by CFUs at 3 days of incubation in THP-1 macrophages and E) Mycobacterial growth was assessed by rRNA 16S expression at 1 day of incubation in MDMs. Data represent the mean ± SE (n = 3). *p < 0.05, significant difference of each treatment (W, SB or medium) in comparison to cells treated with NDGA alone.

through the PI3K/AKT/mTOR autophagy pathway in THP-1 cells and MDMs and that autophagy is related to cytotoxic mechanisms against Mycobacteria. It is well known that autophagy is not the only bactericidal mechanism that is inhibited by wortmanin and SB, because both compounds inhibit phagosome lysosome fusion [51-53]. In fact, cells treated with these inhibitors and NDGA reached a higher mycobacterial load compared to non-treated cells, while the LC3II puncta counts were reestablished to the control level (cells cultured in medium). The exacerbated bacterial growth detected in our results could also be due to the blockade of phagosome maturation by wortmannin and SB in addition to autophagy inhibition.

Furthermore, in a breast cancer model, it has been demonstrated that NDGA inhibits one of the main regulatory mechanisms of autophagy such as the mammalian target of rapamycin complex 1 (mTORC1) through disruption of mTOR–Raptor complex preventing cell proliferation [54]. Moreover, it has been reported that the main NDGA antioxidant properties rest on the nuclear translocation of Nrf2 and consequently the release of Kelch-like ECH-associated protein 1 (Keap1) [55-56], and the interaction of p62 with Keap1 increases the autophagic flux and that the downregulation of Keap1 decreases the autophagy levels [57]. Therefore, it is possible that NDGA exerts different mechanisms involved in autophagy induction in macrophages.

In addition, we observed that NDGA treatment decreases TNF-α production and that the inhibition with wortmannin and SB did not restore the effect caused by NDGA. In this regard it has been reported that the treatment with NDGA exerts an anti-inflammatory effect in THP-1 cells by decreasing lipoxygenase activity, which in turn decreases TNF-α production [20]. Therefore we speculate that reduction of TNF-α by NDGA could be a mechanism independent of autophagy induction and could prevent the exacerbation of inflammation and contribute to the elimination of intracellular bacteria.

Our study suggests the potential use of NDGA as adjunctive therapy for tuberculosis treatment. Regarding the NDGA toxicity in humans, this compound has been tested for prostate cancer therapy; the daily and continuous administration of NDGA (≤2250 mg) for 28 days was reasonably well tolerated, only some patients presented fatigue, diarrhea, nausea, dizziness and headache. In contrast, the continuous administration of NDGA for more of 18 weeks was slightly toxic, 60% of patients showed high transaminase levels, a marker of liver damage [58-59]. Additional studies are required to determine the optimum concentration to obtain the desirable immunomodulatory effects in tuberculosis treatment without the possible adverse effects.

In conclusion, high concentrations of NDGA and α-mangostin inhibited the growth of *M. tuberculosis* and low concentrations simultaneously prevented intracellular bacterial growth in human macrophages. These findings allow us to suggest that these compounds could be used in combination with INH and RIF and may contribute to the treatment of drug-resistant tuberculosis.

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