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A new chemotherapeutic approach using doxorubicin simultaneously with geopropolis favoring monocyte functions

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ABSTRACT

Aims: Chemotherapy has been widely used to treat cancer although it may affect non-target cells involved in the immune response. This work aimed at elucidating whether the chemotherapeutic agent doxorubicin in combination with geopropolis produced by *Melipona fasciculata* Smith could affect nontumor immune cells, evaluating their immunomodulatory effects on human monocytes.
Main methods: Cell viability, expression of cell markers (HLA-DR, TLR-2, TLR-4, C80 and CD40), cytokine production (TNF-α, IL-1β, IL-6 and IL-10), intracellular pathways (NF-κB and autophagy), the microbicidal activity of monocytes and hydrogen peroxide (H_2O_2) production were analyzed.
Key findings: Data showed that doxorubicin + geopropolis diminished IL-6 secretion, stimulated TNF-α and IL-10 production, TLR-4 and CD80 expression, NF-κB and autophagy pathway, as well as the bactericidal activity.
Significance: Our findings revealed a new chemotherapeutic approach using doxorubicin simultaneously with geopropolis without affecting human monocytes viability and exerting immunomodulatory effects, favoring cell functions. While doxorubicin altered some immunological parameters, the addition of geopropolis compensated some changes.

1. Introduction

The American Cancer Society predicted about 1.6 million new cases of cancer in 2017 and 600,000 deaths [1]. Although chemotherapeutic agents (e.g. carboplatin, methotrexate, doxorubicin, and others) have been widely employed, they may cause side effects due to lack of selectivity between normal and tumor cells [2,3]. Regarding the immune system, doxorubicin may cause toxicity to the blood progenitors and changes in cell markers, impairing monocyte functions such as phagocytosis and hydrogen peroxide (H_2O_2) release. Monocytopenia is one of the undesired side effects of chemotherapy [4].

Monocytes can scour the blood vessels, migrate across the endothelium and enter the tissues, differentiating into macrophages in response to inflammatory or infectious signals. Monocytes may acquire antigen-presenting capability and link the innate and adaptive responses by processing antigens, expressing human leukocyte antigen molecules, as well as co-stimulatory molecules for T cell activation. During chemotherapy, monocytopenia may compromise the immune response, although it allows clinical oncologists and physicians to select an appropriate chemotherapy protocol [4–6].

Previous findings of our group revealed that the cytotoxic action of chemotherapeutic agents against tumor cells may be potentiated in combination with geopropolis [7]. Although propolis produced by Africanized bees has been widely investigated, geopropolis has been the focus of recent research. Geopropolis is produced by stingless bees and contains wax, plant exudates, pollen, some gland secretion and the typical presence of soil. Meliponinae is a large group of stingless bees found in tropical and subtropical regions of the planet. Geopropolis produced by *Melipona fasciculata* Smith in Maranhão State, Brazil, has been shown to exert cytotoxic effects against tumor cells, antimicrobial action and immunomodulatory effects [8–10]. Doxorubicin in combination with geopropolis exerted a synergistic effect on HEp-2 cells, suggesting that this combination could be a novel clinical chemotherapeutic approach for cancer treatment [7].

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Since doxorubicin + geopropolis exerted cytotoxic effects on tumor cells, a possible effect of this combination on the innate immunity could take place. The combination doxorubicin + geopropolis seemed to be feasible, because it exerted no cytotoxic/cytostatic effects on THP-1 cells and presented an immunomodulatory action [11]. However, considering a high variability among individuals undergoing chemotherapy, the purpose of this work was to elucidate the effects of doxorubicin + geopropolis on monocytes isolated from healthy donors by assessing cell viability, expression of cell markers (HLA-DR, TLR-2, TLR-4, CD80 and CD40), cytokine production (IL-1β, TNF-α, IL-6, IL-10), intracellular pathways (NF-κB and autophagy), the microbicidal activity against *Candida albicans*, *Escherichia coli* and *Streptococcus mutans,* and H_2O_2 production.

2. Materials and methods

2.1. Doxorubicin, geopropolis and their combination

Doxorubicin (Bergamo, Taboão da Serra, SP, Brazil) was diluted in RPMI 1640 culture medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal calf serum (complete medium) to obtain 1 μM.

Geopropolis was produced by *M. fasciculata* Smith in Palmeirândia (2° 39′ S, 44° 55′ O), Maranhão State, northeast Brazil and kept at 4 °C before extraction [8]. A sample (30 g) was ground using an electric blender and macerated in 70% ethanol (100 ml) at room temperature under moderate shaking. After 24 h, the extract was filtered and its dry weight was calculated (9.6 mg/ml). Geopropolis was diluted in RPMI 1640 to obtain 25 μg/ml.

The combination doxorubicin + geopropolis was prepared using 1 μM and 25 μg/ml, respectively. These concentrations were established according to previous assays standardized in our laboratory [7].

2.2. Human monocytes culture

Blood samples (20 ml), obtained from 10 healthy adult volunteers, were collected and placed in sterile tubes containing 200 μl of heparin. Subsequently, peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque Plus (GE, Boston, MA, USA) gradient separation by centrifugation for 30 min at 400 × *g*. The interface layer of PBMC was aspirated and washed twice with RPMI 1640 at 300 × *g* for 10 min. Thereafter, the supernatant was discarded and the pellet of cells resuspended in 1 ml of complete RPMI medium. Monocytes (1×10^6 monocytes/ml) were incubated at 37 °C and 5% CO_2 for 2 h. The purity of the working population was analyzed by flow cytometry (Fig. S1). Non-adherent cells were removed and monocytes were incubated with doxorubicin, geopropolis or their combination for performing the experiments. As a baseline control, the cells were incubated with complete medium alone. Lipopolysaccharide (LPS 10 μg/ml – Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control because it activates the transcription factor NF-kB which in turn migrates to the nucleus to induce the expression of several genes involved in the immune response.

In order to analyze the effects of doxorubicin and geopropolis alone or in combination on cell viability and cytokine production, cells were incubated with the stimuli simultaneously or not with LPS for 18 h, since this period of time may accommodate the time lag between the addition of the stimuli and cell response in vitro.

This study was approved by the Ethics Committee of Botucatu Medical School (CAAE: 6827 1617.8.0000.5411). An informed consent was signed by all the blood donors.

2.3. Monocytes viability

To verify a possible cytotoxic effect of doxorubicin, geopropolis and their combination, monocyte viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT – Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay. After 18 h incubation with stimuli in absence or presence of LPS (10 μg/ml), culture medium was replaced by 300 μl of MTT (1 mg/ml) and incubated for 3 h. Afterwards, MTT was removed and 200 μl of dimethyl sulfoxide (DMSO – Sigma-Aldrich, St. Louis, MO, USA) were added. The optical density (O.D.) was read at 540 nm and the percentage of monocyte viability was calculated by the formula: (O. D. test / O. D. control) × 100.

2.4. Cell markers determination by flow cytometry

In order to evaluate the effects of stimuli on antigen recognition (TLR-2 and TLR-4), presentation (HLA-DR), and on costimulatory molecules (CD80 and CD40) expression, monocytes (1×10^6 cells/ml) were distributed (500 μl) into polystyrene tubes (Becton Dickinson, San Jose, CA, USA) for cytometric analysis. Cells were evaluated after 18 h incubation with geopropolis, doxorubicin and their combination. Cells were washed and incubated for 30 min in the dark at 4 °C with FITC, PE and APC-conjugated monoclonal antibodies (0.5 μg/ml – Biolegend Inc., San Diego, CA, USA), as follows: anti-TLR-2-FITC (clone TL2.1), anti-TLR-4-PE (clone HTA125), anti-HLA-DR-FITC (clone L243), anti-CD80-PE (clone 2D10), anti-CD40-APC (clone 5C3) and anti-CD14-PerCP/Cy5.5 (clone HCD14). Isotypic antibodies labeled with fluorochromes of the respective tests (FITC, PE, APC and PerCP/Cy5.5) were used as isotypic control. After incubation (4 °C, 30 min), cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A CellQuest Software (Cell Quest Software, San Jose, CA, USA) was used to analyze 30,000 events. Cell gates were established on the basis of size parameters (FSC) and granularity (SSC). Results were expressed as percentage of cells bound with antibodies, obtained for the gated CD14+ cells (Fig. S2).

2.5. Cytokine quantification

Cytokines were evaluated to observe a pro- or anti-inflammatory profile after monocytes incubation with doxorubicin, geopropolis and their combination. TNF-α, IL-1β, IL-6 and IL-10 production by monocytes was analyzed by the enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.6. NF-κB and LC3: RNA extraction, gene expression, Western blot and immunofluorescence

Since cytokines production is related to intracellular pathways, two possible targets were analyzed: NF-κB and autophagy pathway. Total RNA was obtained from the upper aqueous phase from the lower organic phase of TRIzol by the TRIzol-based method. Total RNA was extracted according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Subsequently, RNA integrity and concentration were measured spectrophotometrically using a Synergy HTX Multi-Mode Microplate Reader with Take3 Trio Micro-Volume plate accessories (BioTek, Winooski, VT, USA).

2.6.1. NF-κB and LC3 gene expression analysis

Quantitative polymerase chain reaction (qPCR) was performed using a QuantStudio® 3 Real-Time PCR System to assess changes in mRNA expression in the genes reported in Table 1. Total RNA was extracted from cells with Ambion TRIzol Reagent (Life Sciences – Fisher Scientific Inc., Walthan, MA, USA) and treated with DNase I (Invitrogen, Carlsband, CA, USA). cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. qPCR was carried out in a total volume of 10 μl, containing PowerUp™ SYBR™ Green Master Mix 2× (5 μl) (Applied Biosystems, Foster City, CA, USA), 0.4 μM of each primer, 100 ng of cDNA and nuclease free H_2O. Fold changes were analyzed using the comparative CT method

Table 1
Genes and primer sequences.

Gene (ID)	Primer 5′- 3′ sequence forward reverse
NF-κB (4790)	CACCTGTTCCAAAGAGCACC GGTTCAGGAGCTGCTGAAAC
LC3 (84557)	CTTCTGAGCCAGCAGTAGGG GAGGGACAACCCTAACACGA
β-actin (60)	ACAGAGCCTCGCCTTTGC GCGGCGATATCATCATCC
GAPDH (2597)	GACTCATGACCACAGTCCATGC AGAGGCAGGGATGATGTTCTG
18S rRNA (106632259)	CGGACAGGATTGACAGATTGATAGC TGCCAGAGTCTCGTTCGTTATCG

The reaction conditions were: 95 °C – 15 s; 65 °C – 30s; 72 °C – 30s.

(ΔΔCt) normalizing to β-actin, GAPDH and 18S rRNA expression and comparing to static conditions as a reference.

2.6.2. Western blot analysis

After 18 h incubation, blood monocytes cells were rinsed with PBS pH = 7.4 and lysed for 1 h on ice in RIPA buffer supplemented with protease inhibitors (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. Subsequently, protein extracts were cleared by centrifugation. After protein concentration and quantification by *Lowry Protein Assay* (*Bio-Rad, Hercules, CA, USA*), an equal amount of each protein (75 μg) was separated by SDS-PAGE and transferred to PVDF-membrane (Millipore, Bedford, MA, USA). After blocking with 1% fat-free dried milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST), the membrane was exposed overnight at 4 °C to primary antibodies detecting phospho-IκBα (1/1000) and β-actin (1/1000) as loading control. Revelation was performed by incubating the membrane for 1 h at room temperature with secondary horseradish peroxidase-conjugated and anti-mouse (1/5000), followed by ECL detection (Abcam, Cambridge, UK). Band intensities were thereafter quantified using ImageJ software and graphics were prepared using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

2.6.3. Immunofluorescence

Cells were grown on glass coverslips placed in the peripheral ring of the culture. Afterwards, cells were washed with PBS, fixed in PBS-*p*-formaldehyde (4% v/v) for 1 h, permeabilized in PBS containing 0.2% Triton-X 100 and 1% BSA at 37 °C for 1 h and stained with the appropriate specific primary antibodies anti-NF-κB and anti-LC3, at the concentration recommended by the manufacturer. Then, after washing with PBS to remove the primary antibody for fluorescence analysis, the cells were stained with Alexa Fluor 594 anti-rabbit (LC3) or Alexa Fluor 488 anti-mouse (NF-κB) IgG antibody for 1 h. For actin cytoskeleton rearrangement analysis, the cells were incubated for 40 min with 4 mg/ml, Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA). Cells were washed and coverslips were mounted on glass slides using Fluorosield with DAPI (Sigma-Aldrich, St. Louis, MO, USA), using an inverted laser scanning confocal microscope (Leica TCS SP5). The quantitation of nuclear NF-κB and cytoplasmic LC3 was obtained by the Lasaf software.

2.7. Microbicidal activity of monocytes

To observe the fungicidal and bactericidal activity of monocytes after incubation with doxorubicin, geopropolis and their combination, cells were distributed in the culture plates (1×10^6 cells/ml) and incubated with doxorubicin, geopropolis and their combination for 18 h at 37 °C. After, supernatants were removed and cells were challenged with *Candida albicans* (SC 5314), *Escherichia coli* (ATCC 43895) and *Streptococcus mutans* (CCT 3440), in the ratio of 1:5 monocyte/fungi or bacteria. Microorganisms incubated only with culture medium and plated were used as control. After 2 h at 37 °C, supernatants were collected, and the plate wells were subjected to several washes with ice-cold saline buffer. Same procedures were performed with control. The total volume obtained by removing the supernatant and the washes was 10 ml. From this volume, 20 μl were plated in brain heart infusion (BHI – Oxoid, Walthan, MA, USA) agar to evaluate the fungal and bacterial growth and to calculate the microbicidal activity of monocytes. After 48 h, the colony forming units (CFU) were counted and the percentage (%) of microbicidal activity was calculated using the following formula: % microbicidal activity = [1 − CFU treated cultures / CFU control] × 100.

2.8. Hydrogen peroxide production

In order to verify whether reactive oxygen species production was involved in the microbicidal activity of monocytes, dichlorofluorescein (DCF) was used as an indicator of the intracellular formation of ROS. Monocytes (1×10^6 cells/ml) were incubated with medium alone (control), geopropolis, doxorucibin and their combination. After 18 h, 5.3 mM of phorbol 12-myristate 13-acetate (PMA) was added for 15 min. Cells were harvested, centrifuged and washed with 1× PBS. DCF-DA (5 μM) was added for 45 min, the cells were washed with PBS and centrifuged, the supernatant was discarded and the cell pellet was resuspended. DCF fluorescence intensity was analyzed at an excitation wavelength of 480 nm and fluorescence emission was measured in FL-1 channel at 530 nm using the FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer. For each analysis, 40,000 events were recorded. Cell gates were established on the basis of size parameters (FSC) and granularity (SSC). Results were expressed as median fluorescence intensity (MFI) of DCF positive cells.

2.9. Statistical analysis

Data were analyzed using the GraphPad Prism 5 software (Graph Pad, San Diego, CA, USA). Analysis of variance (ANOVA) and Dunnett's test were employed. Data were expressed as means ± standard-deviation of 10 individuals and differences were considered significant at $P < 0.05$.

3. Results

3.1. Cell viability

The viability of human monocytes after incubation for 18 h with geopropolis and doxorubicin alone or in combination (doxorubicin + geopropolis) was assessed by MTT assay, in the absence or presence of LPS. The stimuli did not exert cytotoxic effects on monocytes and cell viability was ≥90% (Fig. 1).

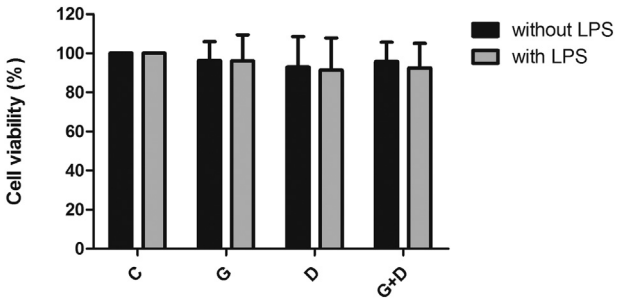

Fig. 1. Viability (%) of human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM) or their combination (G + D) after 18 h, in the absence or presence of LPS (10 μg/ml). Data represent means ± standard-deviation ($n = 10$).

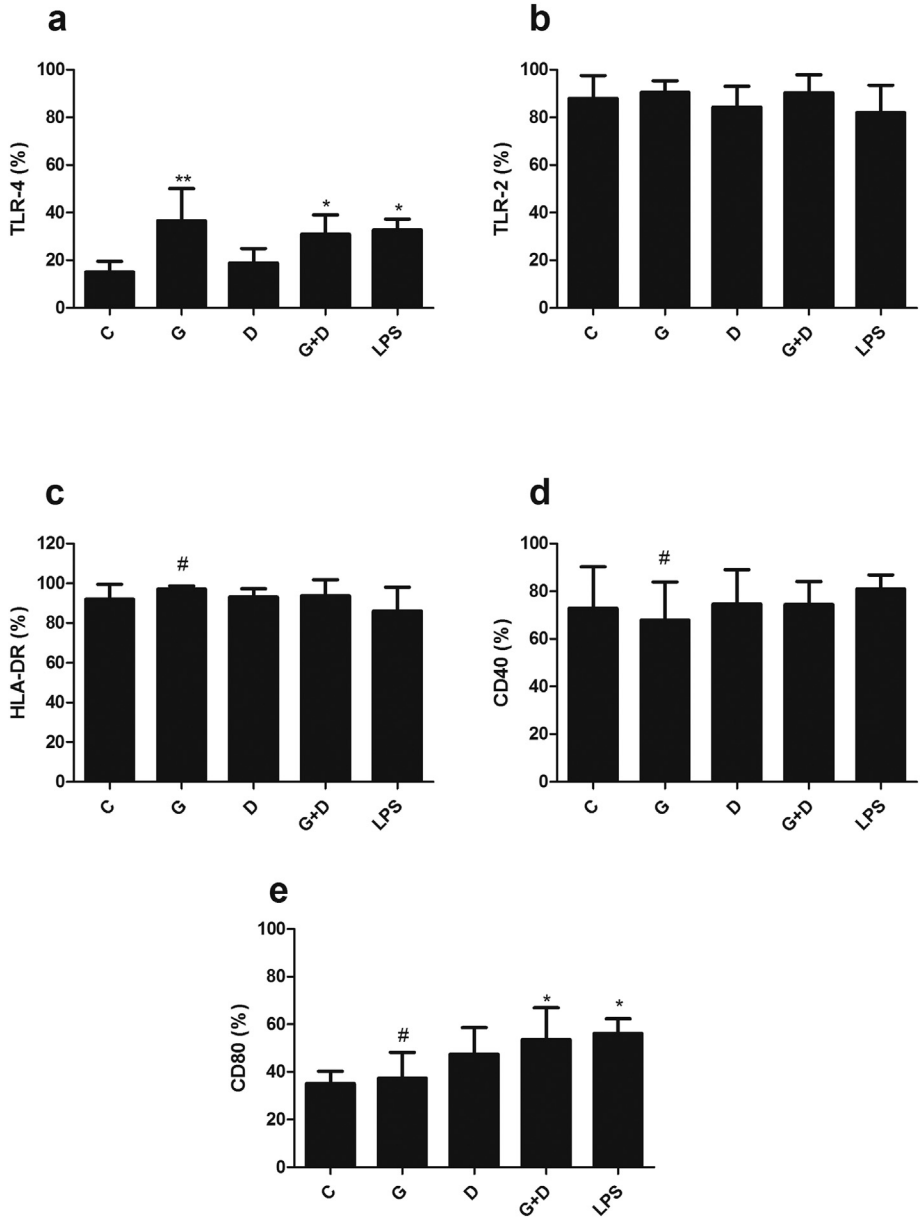

Fig. 2. Percentage (%) of (a) TLR-4, (b) TLR-2, (c) HLA-DR, (d) CD40, and (e) CD80 expression by human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM), their combination (G + D) or positive control (LPS – 10 μg/ml) by 18 h. Data represent means ± standard deviation ($n = 10$). Significantly different from control: *($P < 0.05$) and **($P < 0.01$); significantly different from LPS: $^{\#}$($P < 0.05$).

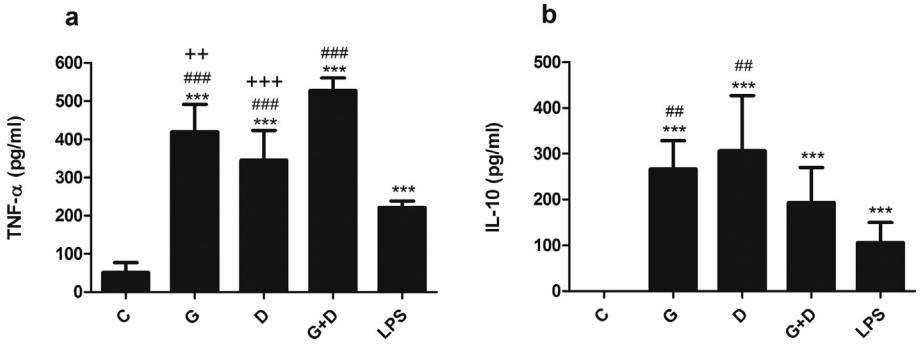

Fig. 3. TNF-α (a) and IL-10 (b) production (pg/ml) by human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM) or their combination (G + D) after 18 h in the presence of LPS (10 μg/ml). Data represent means ± standard-deviation ($n = 10$). Significantly different from control: ***($P < 0.001$); significantly different from LPS: $^{\#\#}$($P < 0.01$) and $^{\#\#\#}$($P < 0.001$); significantly different from G + D: $^{++}$($P < 0.01$) and $^{+++}$($P < 0.001$).

3.2. Expression of cell surface markers

Treatment of monocytes with geopropolis and its combination with doxorubicin significantly upregulated TLR-4 expression compared to control. Doxorubicin and geopropolis alone did not affect CD80 expression, whereas their combination increased its expression. The

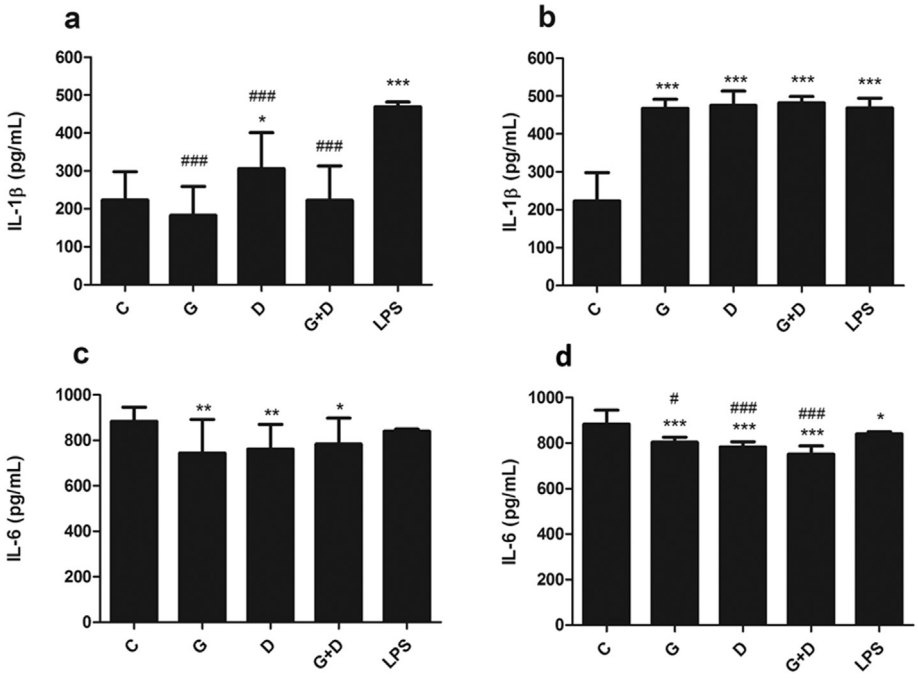

Fig. 4. IL-1β and IL-6 production (pg/ml) by human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM) or combination (G + D) after 18 h in the absence (a, c) or presence (b, d) of LPS (10 μg/ml). Data represent means ± standard-deviation ($n = 10$). Significantly different from control: *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$); significantly different from LPS: $^{\#}$($P < 0.05$) and $^{\#\#\#}$($P < 0.001$).

expression of TLR-2, CD40 and HLA-DR was not altered by the stimuli (Fig. 2).

3.3. Cytokine production

Monocytes were incubated with doxorubicin, geopropolis and their combination for 18 h in the presence or absence of LPS. TNF-α and IL-10 production was not detected when cells were incubated without LPS. In the presence of LPS, doxorubicin and geopropolis alone significantly increased TNF-α and IL-10 production. The combination induced a higher TNF-α production in comparison to the stimuli alone and also stimulated IL-10 production (Fig. 3).

In the absence of LPS, geopropolis decreased IL-1β production, although doxorubicin increased its production compared to control. The combination maintained IL-1β production similar to baseline levels (Fig. 4a). LPS-incubated cells did not show alterations in IL-1β production (Fig. 4b). On the other hand, all stimuli decreased IL-6 production in absence or presence of LPS (Fig. 4c and d).

3.4. RT-qPCR and Western blot for NF-κB and LC3 analysis

NF-κB gene expression was upregulated when cells were treated with doxorubicin, geopropolis or their combination compared to control (Fig. 5a). LC3 expression was significantly stimulated by geopropolis and the combination; on the other hand, doxorubicin exerted a lower effect showing no significant differences compared to control (Fig. 5b). Western blot analysis revealed that geopropolis decreased the phosphorylation of IκBα, but doxorubicin and the combination maintained similar levels compared to control (Fig. 5c and d), while LC3 was not detected.

3.5. Immunofluorescence

Doxorubicin, geopropolis and their combination induced NF-κB translocation to the nucleus (Fig. 6). Regarding autophagy, all cells presented vesicles expressing LC3 protein in the cytosol, but the combination doxorubicin + geopropolis diminished the fluorescence intensity of cytoplasmic LC3 (Fig. 7).

3.6. Microbicidal activity

The fungicidal activity of monocytes treated with doxorubicin and geopropolis alone or in combination, against *C. albicans* did not show significant differences, although a slight decrease was observed after incubation with doxorubicin (Fig. 8).

Concerning the bactericidal activity, doxorubicin significantly decreased the activity of human monocytes challenged with *E. coli*, and the addition of geopropolis compensated doxorubicin action, modulating cell function and increasing the bactericidal activity. There were no significant differences in the bactericidal activity against *S. mutans*, however a discreet increase may be observed when monocytes were treated with the combination (Fig. 8).

3.7. Hydrogen peroxide production

Doxorubicin did not affect H_2O_2 production while geopropolis alone or in combination with doxorubicin inhibited its production by monocytes (Fig. 9).

4. Discussion

Previous findings demonstrated that geopropolis enhanced the action of chemotherapeutic agents against tumor cells [7]; however, it is unknown whether this combination could affect monocytes, since chemotherapy is systemically distributed in the blood stream. Our data showed that doxorubicin, geopropolis and their combination did not affect the viability of human monocytes, permitting the continuity of this work.

A higher TLR expression is important during microbial infections, mainly in individuals with impaired immune system due to chemotherapy [12]. Geopropolis and its combination with doxorubicin upregulated TLR-4 but not TLR-2 expression by monocytes, suggesting that the combination did not affect the recognition of antigens by these cells and the subsequent activation of the adaptive immune response.

Doxorubicin, geopropolis and their combination maintained HLA-DR expression, what is in agreement with previous works using human dendritic cells and propolis produced by Africanized honeybees, suggesting that the stimuli may preserve antigen presentation and promote an efficient immune response [13]. Reduced HLA-DR expression may be

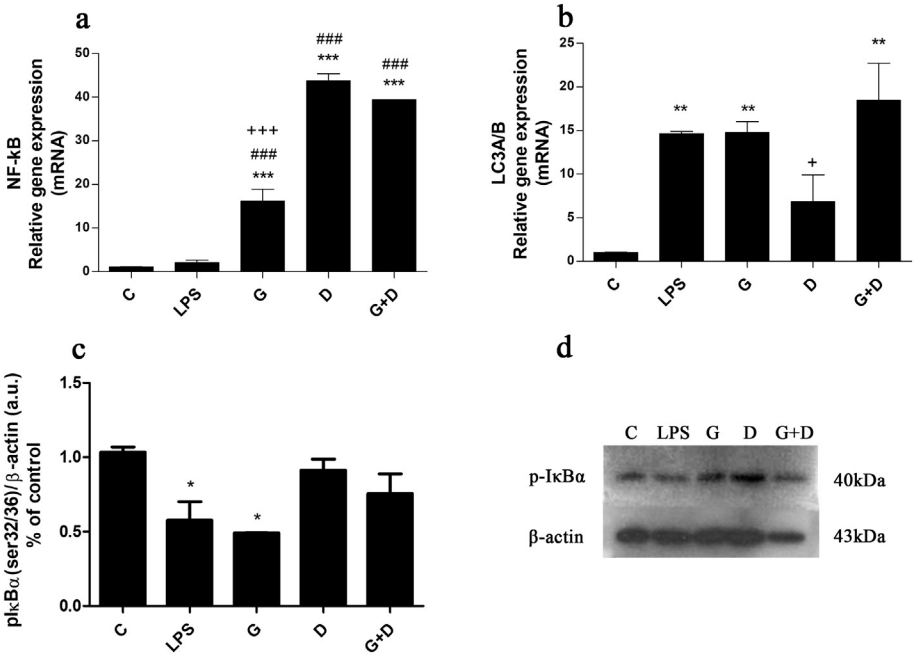

Fig. 5. Relative gene expression of (a) NF-κB and (b) LC3A/B, and (c, d) protein expression of phospho-IκBα (arbitrary units – a.u.) by human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM), combination (G + D) or LPS (10 μg/ml) after 18 h. Data represent mean ± standard-deviation ($n = 10$). Significantly different from control: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$); significantly different from LPS: $^{\#}$($P < 0.05$), $^{\#\#}$($P < 0.01$), $^{\#\#\#}$($P < 0.001$); significantly different from G + D: $^{+}$($P < 0.05$), $^{+++}$($P < 0.001$).

related to the development of non-Hodgkin's lymphoma, and a decreased HLA-DR expression in biopsies of some patients was associated with a lower survival during chemotherapy.

CD40 is expressed by antigen presenting cells during their interaction with T lymphocytes and binds to CD40L, promoting the production of cytokines and the expression of costimulatory molecules during

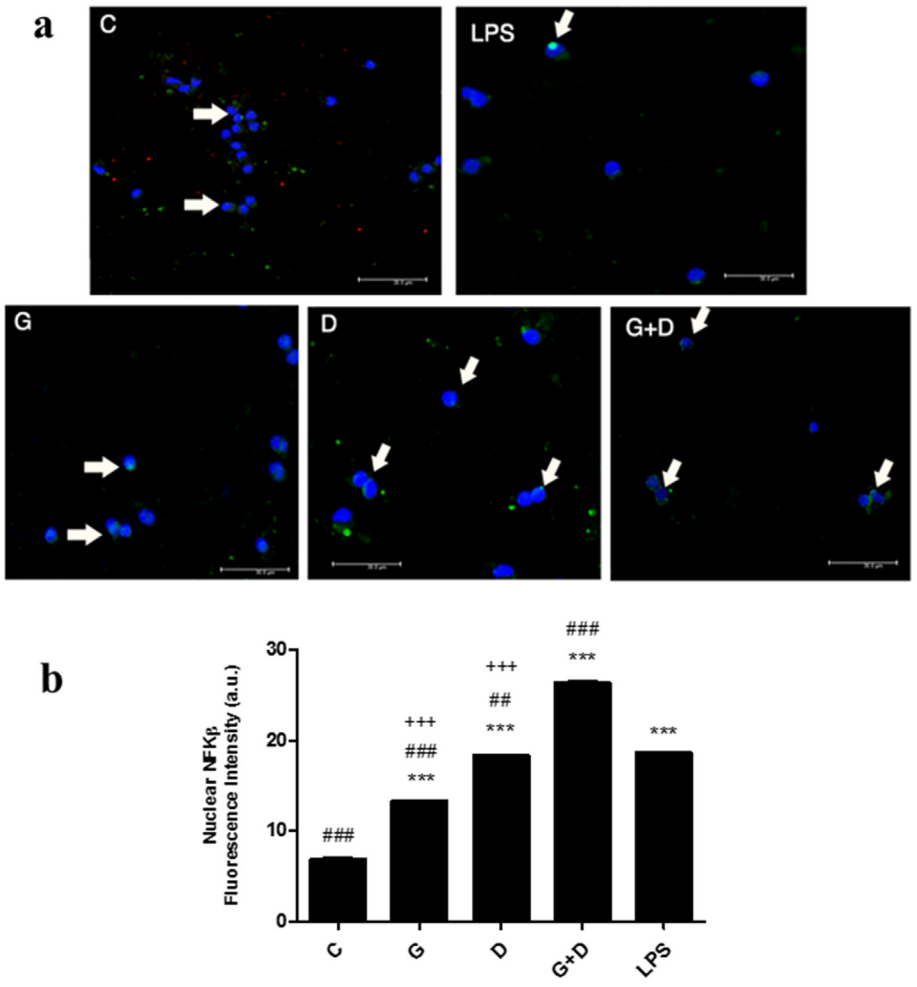

Fig. 6. (a) Laser confocal microscopy analysis of human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM), combination (G + D) or LPS (10 μg/ml) for 18 h. Cells were incubated with specific antibody, followed by staining with Alexa Fluor 488 goat anti-mouse IgG antibody (green) for NF-κB. The nuclei were stained with DAPI (blue). Bar = 30 μm. (b) Fluorescence intensity of nuclear NF-κB (arbitrary units – a.u.). Data represent mean ± standard-deviation ($n = 10$). Significantly different from control: ***($P < 0.001$); significantly different from LPS: $^{\#\#}$($P < 0.01$), $^{\#\#\#}$($P < 0.001$); significantly different from G + D: $^{+++}$($P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

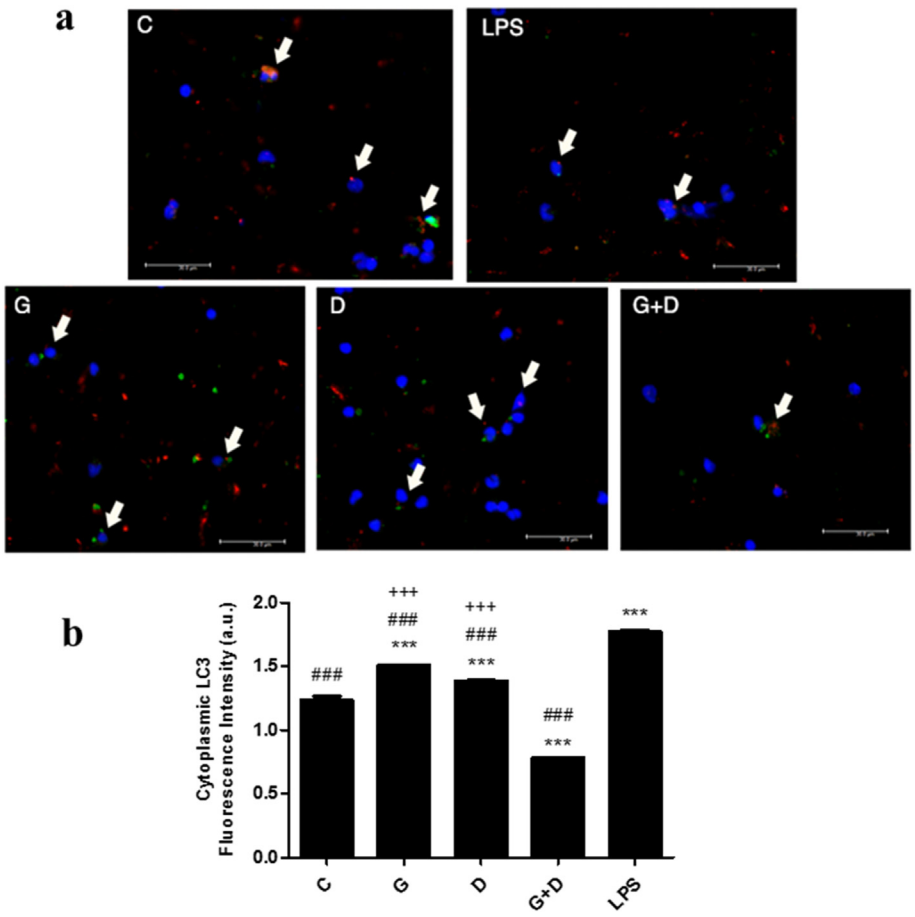

Fig. 7. Laser confocal microscopy analysis of human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM), combination (G + D) or LPS (10 μg/ml) for 18 h. Cells were incubated with specific antibody, followed by staining with Alexa Fluor 594 goat anti-rabbit IgG antibody (red) for LC3 and Alexa Fluor 488 (green) for β-actin. The nuclei were stained with DAPI (blue). Bar = 30 μm. (b) Fluorescence intensity of cytoplasmic LC3 (arbitrary units – a.u.). Data represent mean ± standard-deviation ($n = 10$). Significantly different from control: $^{***}(P < 0.001)$; significantly different from LPS: $^{\#\#\#}(P < 0.001)$; significantly different from G + D: $^{+++}(P < 0.001)$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inflammatory responses and in the cytotoxic response against tumor cells [14]. Here, doxorubicin, geopropolis and their combination did not affect CD40 expression. The combination doxorubicin + geopropolis increased CD80 expression, favoring the activation of T lymphocytes. Overall, data showed that the combination may not impair antigen recognition and costimulatory molecule expression.

Regarding cytokine production, geopropolis induced both TNF-α and IL-10 production in human monocytes [8]. Further, our group demonstrated that geopropolis also increased TNF-α production by THP-1 cells [11]. In this work, doxorubicin + geopropolis stimulated TNF-α and IL-10 production in human monocytes, what indicates the immunostimulant profile of the combination. TNF-α can act on caspase-8 pathway in tumor cells, altering mitochondrial membrane potential with a consequent activation of apoptotic complex, resulting in cell death [15].

Although doxorubicin + geopropolis increased IL-10 production, they also increased TLR-4 expression, suggesting a differential activation of IL-10 and TLR-4 pathways by our stimuli. IL-10 is well known for its anti-inflammatory action but it can also suppress tumor growth, modulating apoptosis, inhibiting angiogenesis and downregulating MHC class I in cancer cells enhancing natural killer cell-mediated death [16].

IL-1β can act on Th1 cells leading to a cytotoxic action on the neoplastic cells [17]. Since doxorubicin-treated monocytes exhibited an increased IL-1β production and its combination with geopropolis maintained its baseline level, it may reflect an inflammatory character of the chemotherapeutic agent. IL-6 exerts both pro- and anti-inflammatory action and its production is related to the activation of NF-κB or AP-1 [18]. Here, the combination doxorubicin + geopropolis inhibited IL-6 production by monocytes, what may be due to the increased IL-10 production. Furthermore, an upregulated IL-6 production has been associated with tumor growth and chemotherapy resistance.

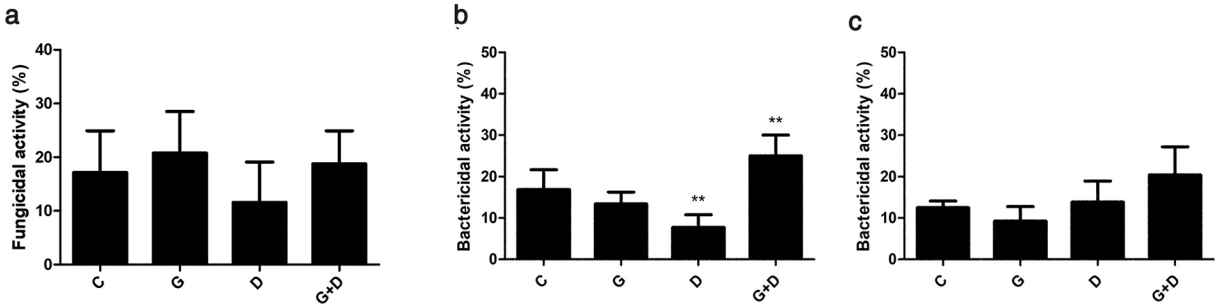

Fig. 8. Percentage (%) of the fungicidal and bactericidal activity of human monocytes incubated with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM) or their combination (G + D) for 18 h, and challenged with (a) *Candida albicans* (monocytes/fungi 1:5), (b) *Escherichia coli* or (c) *Streptococcus mutans* (monocytes/bacteria 1:5) for 2 h. Data represent means ± standard-deviation ($n = 10$). Significantly different from control $^{**}(P < 0.01)$.

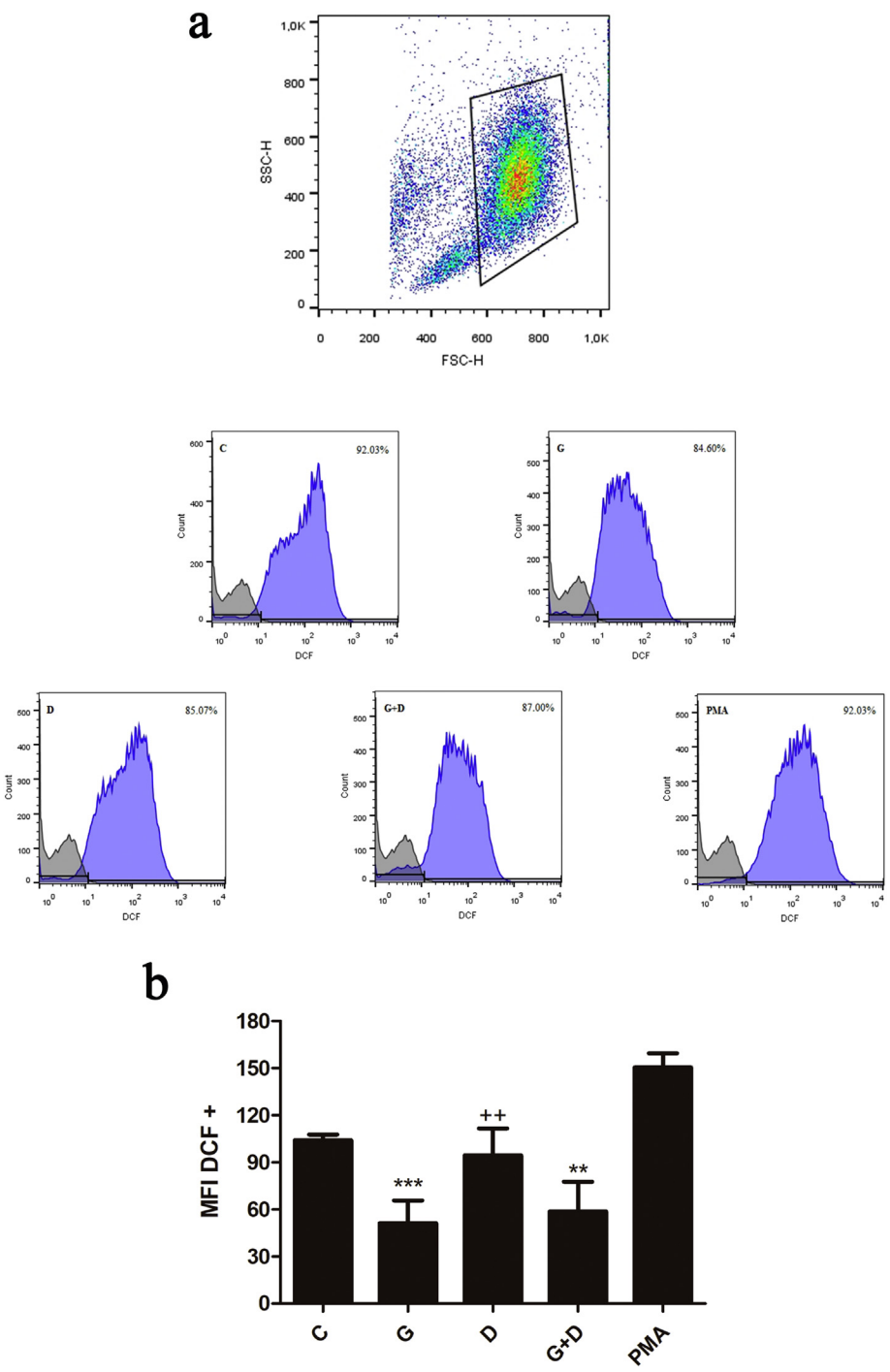

Fig. 9. (a) Representative dot plots of gates based on size parameters (FSC) × granularity (SSC) and histogram (count × DCF). (b) bars graph of DCF+ labeled cells after incubation with RPMI 1640 (control – C), geopropolis (G – 25 μg/ml), doxorubicin (D – 1 μM), combination (G + D) and PMA (5.3 mM) for 18 h. The gate of DCF+ cells was delineated in negative control (autofluorescent). Data represent means ± standard-deviation ($n = 10$). Significantly different from control **($P < 0.01$), ***($P < 0.001$); significantly different from G + D: $^{++}$($P < 0.01$).

Indeed, chemically-induced mice to develop hepatocellular carcinoma exhibited an increased IL-6 concentration [19]. A reduced IL-6 production was seen in monocytes incubated with the combination, what suggests that it could alter the tumor growth and probably prevent chemotherapy resistance in patients.

The combination doxorubicin + geopropolis may act in different intracellular pathways in human monocytes, since TNF-α production is related to NF-κB activation, IL-10 is under STAT3 control, IL-1β is related to inflammasome or autophagy pathways, and IL-6 to NF-κB and AP-1 pathways [20–23]. Thus, two possible targets were further analyzed: NF-κB and autophagy pathway.

In cancer cells, the combination doxorubicin + resveratrol led to apoptosis mediated by downregulation of genes and proteins associated with inflammation (NF-κB, COX-2) and autophagy (LC3B, Beclin-1), suggesting the crosstalk of signaling pathways associated with chronic inflammation, autophagy and apoptosis during breast cancer pathogenesis [24]. In monocytes, our data showed that doxorubicin + geopropolis exerted a stimulant effect inducing both NF-κB expression and TNF-α production. Moreover, doxorubicin + geopropolis upregulated LC3 expression in monocytes.

Autophagy is important during monocyte-macrophage differentiation and has been proposed to protect from various diseases including cancer [25]. Tacar and Dass reported that doxorubicin-induced autophagy could be beneficial for a good prognosis of cancer patients, since this pathway is responsible for destroying damaged organelles and inducing tumor cell death [26].

Although the western blot assay did not show high levels of phospho-IκBα or quantitative results of LC3 proteins, the immunofluorescence staining revealed the presence of NF-κB in the nucleus and the formation of LC3 containing vesicles, demonstrating that these intracellular pathways are indeed being affected by doxorubicin, geopropolis and their combination. Doxorubicin + geopropolis upregulated TLR-4 expression, which is responsible for inducing NF-κB activation and autophagy pathway [27,28]. Thus, the combination may probably induce NF-κB and autophagy through interaction with TLR-4, showing its activator profile.

The ability of monocytes to recognize pathogens is one of the first defenses against invading microorganisms. Halder et al. demonstrated that monocytes respond to microorganisms via phagocytosis, decondensation of nuclear DNA and release of DNA in the form of monocytic extracellular traps called “MoETs” [29]. Regarding the fungicidal activity, there was no significant difference after monocytes incubation with doxorubicin, geopropolis and their combination and challenge with *C. albicans*. Although a non-significant decreased fungicidal activity may be observed in doxorubicin-treated monocytes, it seemed to be compensated by the combination with geopropolis. Blood mononuclear cells treated with microspheres containing propolis produced by Meliponinae bees were efficient in phagocytosis and superoxide anion production in response to *C. albicans* [30]. The combination doxorubicin + geopropolis stimulated significantly the bactericidal activity of monocytes against *E. coli,* what may help such cells to combat one of the most common bacterial infections among patients undergoing chemotherapy [31]. Moreover, the combination upregulated TLR-4 expression, that could be related to the increased bactericidal activity of monocytes.

Patients undergoing chemotherapy have a higher index of caries and periodontal diseases. Here, no significant differences were seen in the bactericidal activity against *S. mutans*, although a mild increase was observed when monocytes were treated with the combination. Previous works of our group demonstrated that propolis produced by Africanized honeybees increased the bactericidal activity of monocytes and dendritic cells against *S. mutans* [13,32].

ROS levels could account for a more efficient antimicrobial effect, since lower bactericidal and fungicidal activities of immune cells may be associated to decreased ROS levels. Doxorubicin did not affect H_2O_2 production by monocytes while geopropolis alone or in combination diminished it. Thus, no correlation could be seen between such parameters, because doxorubicin decreased the microbicidal activity of monocytes and the addition of geopropolis compensated its action. These findings suggested that non-oxidative mechanisms such as increased lysosomal content and acidification of phagosomes may be involved in the killing of microorganisms by these cells, increasing their microbicidal activity. Regarding tumor cells, ROS generation may both contribute to kill them or favor tumor development. In vivo, monocytic-myeloid suppressor cells may exert immunosuppressive effects due to ROS production, among other factors. The reaction of superoxide anion with nitric oxide generates peroxynitrite, which may inhibit T cells by nitrating T cell receptors (TCRs), affecting the binding to antigenic peptides and MHC molecules in tumor cells and blocking T cell migration by nitrating T cell–specific chemokines, conferring a pro-tumor function [33], what indicates that ROS generation has ambiguous effects depending on the context.

Taking together, the combination doxorubicin + geopropolis was cytotoxic to tumor cells as previously reported but not to human monocytes. Although the cells were probably already stimulated, the combination diminished IL-6 secretion and stimulated TNF-α and IL-10 production, TLR-4 and CD80 expression, NF-κB and autophagy pathways. Doxorubicin altered some immunological functions but the addition of geopropolis seemed to compensate these alterations, as observed for IL-1β production and the microbicidal assay.

5. Conclusion

The combination doxorubicin + geopropolis did not affect human monocytes viability and exerted immunomodulatory effects, favoring cell functions. While doxorubicin altered some immunological parameters, the addition of geopropolis compensated some changes. This work was carried out in vitro, what may be considered a limitation of this study; however, it opens perspectives for future studies in vivo as a novel chemotherapeutic approach using doxorubicin simultaneously with geopropolis preserving monocyte functions.

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Disclosure statement

The authors declare that they have no conflicts of interests.

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