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Extracts of *Luffa Operculata* Downregulate some Functions of Human Circulating Leukocytes

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Abstract: ***Background: Luffa operculata is one of the most popular medicinal plants used in South America, where it has been proposed as a low-cost alternative for the treatment of rhinosinusitis, although the mechanisms underlying its anti-inflammatory, immunosuppressive, and also anti-tumor effects are unknown. This study aimed to determine the range of cytotoxic concentrations of aqueous and ethanolic extracts of L. operculata for human circulating leukocytes, and evaluate the effects of non-cytotoxic doses on some functions of leukocytes from patients with rhinosinusitis and healthy controls. Methods: Samples of EDTA-anticoagulated peripheral venous blood were drawn from healthy volunteers and asymptomatic patients with a clinical history of chronic rhinosinusitis. Using nontoxic concentrations of ethanolic and aqueous extracts the effects on cell PHA-induced proliferation, respiratory burst and cytokines were studied by flow cytometry. Results: Non-cytotoxic doses of both, aqueous and ethanolic extracts of L. operculata inhibited the PHA-induced activation and proliferation of lymphocytes, and significantly reduced the accumulation of IL-2, IL-4, IL-8, and TNF-α in the culture supernatants. Also, both plant extracts inhibited the PMA-induced respiratory burst in neutrophils and monocytes in a dose-dependent manner, being monocytes the cells more sensitive. On the other hand, there were no differences in the effects of plant extracts between cells from healthy donors and patients with rhinosinusitis. Conclusion: In this study, cytotoxic and anti-inflammatory effects of L. operculata could be independently attributed to different concentrations of the aqueous and ethanolic plant extracts. Results pointed L. operculata as a negative regulator of the proliferation and synthesis of proinflammatory cytokines by human lymphocytes, and the respiratory burst of phagocytes.***

Keywords: *Luffa operculate*; human leukocytes; immunomodulation; rhinosinusitis

1. Background

The dried fruit of *Luffa operculate*, a plant of the *Cucurbitaceae* family, is a herbal medicine commonly used in Colombia and Latin America to treat diseases such as rhinosinusitis and rhinitis. The plant is mainly prescribed as an aqueous infusion for being inhaled or directly applied by dripping on mucous membranes. At the 1962 International Homeopathic League Conference in Bad Godesberg, Wilmar Schwabe gave a lecture about the use of *L. operculata* dry fruit infusions for the treatment of sinusitis and similar conditions by natives of some Central American countries. These infusions were reported as homeopathic or adjuvant medicines for the treatment of throat, nose, and ear diseases (Ziegler, 1963, 1964). As an overdose of these plant preparations had caused adverse effects such as atrophy of the affected mucosa, mainly when applied locally by inhalation, Schwabe carried out in London a controlled study with low doses of plant extracts, that was published in the British Homoeopathic Journal in 1965. The authors concluded that although the fruit infusion caused stomach discomfort, mucosal dryness, and other adverse effects, it contained a powerful and quite specific remedy for several catarrhal and sinus conditions (Raeside, 1965).

Later phytochemical analyses revealed that *L. operculata* had triterpenes, glycosides, saponins, free sterols, ceramides, and phenols (Kawahara et al., 2001, 2004; Ogunleye et al., 2020; Takemoto et al., 1984). The most studied triterpenes derived from *L. operculate* include Curcurbutacins, especially cucurbitacin B (CuB) and D (CuD), and isocucurbitacin B. These molecules have been associated with the pharmacological effects of the plant, in terms of cytotoxic, anti-inflammatory, and anti-tumor activities (Guo et al., 2018; Jia et al., 2015; Wang et al., 2014). Some studies reported the presence of 2,3-dicyllophlycylicaric acid, a new molecule with unknown biological activity (Kawahara et al., 2001, 2004; Takemoto et al., 1984). In 2015, CuB was reported to inhibit cell proliferation and synthesis of interleukin 8 (IL-8) by cultured human keratinocytes in an imiquimod-induced skin inflammatory model (Li et al., 2015). Also, CuB reduced inflammation in the lungs of rats in the model of sepsis by ligation and cecal puncture (CLP); animals treated with CuB showed reduced levels of TNF-α and IL-6 in bronchoalveolar lavage (Hua et al.,2017). Also, in a model of *Saccharomyces cerevisiae* under oxidative stress, CuB increased the activity of superoxide dismutase (SOD), decreased the production of reactive oxygen species (ROS), and increased the survival rate of yeasts; authors concluded that CuB could antagonize the oxidative stress (Lin et al.,

2019).

The objective of this research was to evaluate the effects of different concentrations of aqueous and ethanolic extracts of *L. operculata* on human peripheral blood mononuclear cells and determine the feasibility to differentiate the cytotoxic and anti-inflammatory doses of the plant. Then, the effects of non-cytotoxic doses of plant extracts were evaluated on cell proliferation and cytokine synthesis by lymphocytes and the respiratory burst of phagocytes of healthy controls and patients with rhinosinusitis.

2. Methodology

2.1 Preparation of the aqueous and ethanolic extracts of *L. operculata*

Samples of dried *L. operculate* fruits (20 grams), purchased from local medicinal herbs markets, were cut into small pieces using a scalpel. For preparing the aqueous extract, solutes in the fruits were leached by decoction in 350 mL of distilled water for 30 min. After cooling to room temperature, decanting, and filtering through Whatman® No. 3 filter paper, the leachate was centrifuged at 200 x *g* for 5 min to remove particles and aggregates. Subsequently, the extract was aliquoted, filtered (0.2 µm), and stored at -20 °C. For preparing the ethanolic extract, the fruit samples were macerated in 300 mL of absolute ethanol and kept for eight days protected from light. The macerate was decanted, filtered, centrifuged, and aliquoted for storage at -20 °C. Both plant extracts were tested for endotoxin by the *Limulus amoebocyte* lysate assay (Lonza Walkersville, Inc.; Walkersville, MD); the endotoxin concentration was less than 0.03 EU/mL. Several aliquots of the aqueous extract were lyophilized; their dry content was weighed and resuspended at 1 mg/mL distilled water to prepare serial dilutions. The aqueous extract was scanned at wavelengths ranging from 190 to 720 nm, using a Mapada 1800-UV spectrophotometer (Shanghai, China). Then, the optical densities of all dilutions of the aqueous extract were determined at 200, 230, 320, and 380 nm; and a linear regression model was used to calculate the equivalence between dilutions and concentration (mg/mL) to be applied to cell cultures. The ethanolic extract was desiccated and resuspended in 5% ethanol for a similar spectrometric analysis and content quantification.

Subjects
Healthy volunteers and asymptomatic patients with a clinical history of chronic rhinosinusitis participated in the study. They all received appropriate information and signed the consent form approved by the Bioethics Committee of the Faculty of Medicine of the Universidad de Antioquia. A physician validated all clinical information. Individuals who had clinical symptoms or were taking any anti-inflammatory or antihistamine medication were excluded from the study.

Isolation of peripheral blood mononuclear cells
Samples of EDTA-anticoagulated peripheral venous blood (10 mL) were drawn for each test. Blood samples were diluted 1:3 in Dulbecco's Phosphate-Buffered Saline (1X DPBS); then, 6 mL of diluted blood were layered onto 3 mL of Histopaque (1.077 g/L) (Sigma-Aldrich Corporation; St Louis, MO) in 15 mL conical tubes that were centrifuged at 900 x *g* for 30 min. The mononuclear cell layer was removed with a Pasteur pipette and transferred into a new 15 mL tube for washing with 10 mL of DPBS by centrifugation at 600 x *g* for 10 min at room temperature. The supernatant was removed, and the cells were suspended in RPMI 1640 medium (Sigma-Aldrich Corporation) supplemented with glutamine, HEPES, and 10% fetal bovine serum (FBS). Cell viability and numbers were evaluated by trypan blue dye exclusion using a Neubauer's chamber cell counting; cell viability was always higher than 95%.

Cell viability assays
Mononuclear cells (2 x 10^5/ 200 µL per well) were cultured in 96-well plates (Corning; Palo Alto, CA) in RPMI 1640 supplemented with glutamine, HEPES, and 10% FBS at 37 °C, 5% CO_2, and 95% relative humidity for 72 hrs, in the presence or absence of plant extracts (6.89 x 10^{-6} to 2.26 x 10^{-1} mg/mL). The final concentration of the diluent (ethanol or water) was lower than 5%, and its proportion was kept constant for all dilutions tested. Control cultures containing only the diluent of the respective plant extract were set in each experiment. Cells were evaluated at 0, 24, 48 and/or 72 hrs.

Mitochondrial membrane functionality and plasma membrane integrity Mononuclear cells from healthy volunteers were incubated with plant extracts for 0, 24, 48, and 72 hrs; then, cells were stained with 700 nM 3,3'-Dihexyloxacarbocyanine iodide ($DIOC_6$) and 1 µg/mL Propidium Iodine (PI) for 30 min at room temperature. Cells were then acquired on an LSRFortessa™II flow cytometer (BD Biosciences, San Jose, CA) using an excitation wavelength of 488 nm for both fluorochromes and bandpass filters of 530/10 nm and 620/15 nm for detection of the emission wavelengths of $DIOC_6$ and PI, respectively. FACS data were analyzed using the FlowJo Software version 7.6.2 (Tritar Inc., Ashland, OR).

Phosphatidylserine exposure
Phosphatidylserine (PS) exposure on the plasma membrane is a typical apoptosis marker, mediated by effector caspases that cleave enzymes involved in the maintenance of the plasma membrane phospholipid asymmetry (Debret et al., 2003). Therefore, cells previously exposed to the plant extracts for 72 h were washed and suspended in Annexin V binding buffer, stained with FITC-conjugated Annexin V (1µg/mL) and PI (1 µg/mL), and acquired on an LSR Fortessa™II flow cytometer. PI^+ cells were detected at 620/15 nm, and Annexin V^+ cells at 530/10 nm. FACS data were analyzed using the FlowJo.

DNA fragmentation
To determine whether extracts of *Luffa operculata* could induce extensive DNA fragmentation, PBMCs treated with the plant extracts for 72 h, as previously described, were fixed with ethanol at a final concentration of 70% for 12 hrs. Then, cells were washed twice with 0.154 M NaCl and stained with a solution containing 0.37 mg/mL PI, 0.01% v/v Triton X-100, 0.037 mg/mL EDTA, and 30 IU/mL RNase

A. Cells were acquired on an LSRFortessa™II flow cytometer (BD Biosciences) to evaluate the nuclear DNA content. The fluorescent signal of PI was measured at 620 nm, and cell aggregates were excluded by plotting the pulse area (PI-A) against the maximum fluorescence amplitude (PI-W); the threshold value of PI+ cells was defined with unstained cells. For each sample, at least 10,000 events in the single event region were acquired. FACS data were analyzed using the FlowJo.

Cell proliferation

The CFSE dilution assay was used to determine the effect of plant extracts on the proliferation of mononuclear cells, as described elsewhere *(*Lyons, 2000*;* Lyons and Parish, 1994*)* with some modifications. Briefly, 5 x 10^6 PBMCs from healthy controls and patients with chronic rhinosinusitis were suspended in 10 mL of DPBS and mixed with 1 µg/mL CFSE for 10 minutes at 37 °C; then, 1 mL of FBS was added. Cells were washed twice with DPBS and then suspended in 5 ml RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin for 30 min at 37°C; and additional 45-min incubation was carried out to remove the excess CFSE dye. CFSE labeled-PBMCs (1 x 10^5 per 200 µL well) were seeded, in triplicates, in 96-well U-bottom plates (Costar-Corning; Lowell, MA) in RPMI-1640 medium supplemented with 100 IU/mL penicillin, 100 µg/mL of streptomycin, and 10% FBS in the presence or absence of aqueous and ethanolic extracts of *L. operculate*, and stimulated with 2.5 µg/mL PHA for 96 hrs. Cells in triplicates of the cultures were collected, washed with DPBS, stained with fluorochrome-conjugated mouse anti-human mAbs (CD4/APC-700 and CD8/BV450) and PI for 30 min, and acquired (1x10^5) on an LSRFortessa™II flow cytometer. FACS data were used to calculate the percentages of proliferating cells using the FlowJo.

Activation of T-cells

PBMCs were isolated from healthy volunteers to evaluate the effect of the plant extracts on the activation of T-cells. PBMCs (5 x 10^6) were suspended in RPMI-1640 medium supplemented with 10% FBS and cultured at 37 °C, 5% CO_2, and 95 % of relative humidity for 12 hrs under three different conditions: i) in the presence of plant extracts and without PHA; ii) with PHA followed two hours later by the aqueous or ethanolic extracts; and, iii) with the extracts and subsequently with PHA. At the end of the incubation time, cells were stained with fluorochrome-conjugated mouse anti-human mAbs (CD69/ECD, CD3/FITC) for 30 minutes, and washed. Cells (1x 10^5) were acquired on an LSRFortessa™II flow cytometer, and FACS data were analyzed using the FlowJo.

Quantitation of lymphokines

Before harvesting cells for flow cytometry analysis, the supernatants from PHA-stimulated cultures were collected for measuring the concentrations of cytokines. IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 were quantified by Cytometric Bead Array (CBA) (Human Inflammatory Cytokine kit; BD Biosciences, San Jose, CA). Levels of IL-2 and IL4 were measured by CBA Enhanced Sensitivity Flex Set System FlexSET (BD Biosciences) following the manufacturer's recommendations. FACS data were analyzed using the FlowJo.

Respiratory burst

The phagocyte respiratory burst was tested using the dihydrorhodamine 123 (DHR 123) oxidation test according to a protocol previously described (Vowells et al., 1995) with some modifications. Whole blood samples from healthy controls and patients with chronic rhinosinusitis were suspended in 500 µL of RPMI containing the plant extracts and incubated at 37 °C, 5% CO_2, and 95 % of relative humidity for 30 min. Samples were then centrifuged at 900 x *g* for 5 min, the supernatant was discarded, and erythrocytes were lysed with ammonium chloride buffer (1.55 mM NH_4Cl, 10mM NaHCO3, 1mM EDTA) at room temperature for 10 min. After cells were centrifuged at 900 x *g* for 5 min, the supernatant was discarded, the tubes were blotted, and the cells were washed with PBS at 900 x *g* for 5 min. The supernatant was discarded, the cell pellet was suspended in 400 µL PBS containing 2.9 mM DHR 123 and incubated at 37 °C, 5% CO_2, and 95 % of relative humidity for 30 min. Then, cells were stimulated with PMA at a final concentration of 6.5 X $l0^2$ nM and incubated at 37 °C, 5% CO_2, and 95 % relative humidity for 30 minutes. Cells were acquired on an LSRFortessa™II flow cytometer, and the levels of rhodamine 123 were measured at 530/30 nm after excitation at 488 nm. FACS data were analyzed using the FlowJo.

Statistics

Data are presented as mean ± 95% Confidence Interval (95% CI). Independent variables were compared between groups with two-way ANOVA and Bonferroni post hoc tests for contrasting means. All analyses were run in GraphPad Prism version 5 (GraphPad Software; La Jolla, CA) , and statistical significance was established at critical p values ≤ 0.05.

3. Results

This study aimed to discriminate cytotoxic and anti-inflammatory concentrations of *Luffa operculata* extracts through the evaluation of their effects on human circulating leukocytes. The first approach was to determine the concentration range of aqueous and ethanolic plant extracts with cytotoxic activity on human PMBCs. Then, the effects of non-cytotoxic doses of the plant extracts were evaluated on the cell proliferation and production of cytokines by PHA-stimulated lymphocytes and the respiratory burst of PMA-activated phagocytes.

The cytotoxic effect of different concentrations of *Luffa operculata* extracts was evaluated on PMBCs from healthy volunteers. Mitochondrial membrane potential and plasma membrane integrity were assessed by staining with $DiOC_6$ and PI, respectively. Flow cytometric analyses were performed on the mononuclear cell region, which mainly includes lymphocytes, given that the number of monocytes decreased progressively by adhering to the plastic well bottom; this was evidenced under an inverted microscope after removal of cells in suspension and washing of the culture wells (**Figure 1A**). After 24 hrs, cells exposed to either diluent showed homogeneous $DIOC_6$ cell

populations **(Figure 1B)**. In contrast, in cultures with either extract of *L. operculata* two lymphocyte populations ($DIOC6^{high}$ and $DIOC6^{Int}$) were observed, the $DIOC6^{Int}$ cell population suggested some kind of mitochondrial dysfunction **(Figure 1C).** Alterations in the plasma membrane (PI^+ cells) were observed in higher concentrations of *L. operculata* extracts (**Figure 1D)**. In experiments with the aqueous extract, dead PI^+ cells reached 6.47 ± 1.54 % in control cultures (diluent) (**Table 1**); this percentage increased significantly (between 10.70 ± 3.70 and 95.40 ± 1.20 %) when cells were exposed to the aqueous extract at a concentration equal or higher than 4.41 x 10^{-4} mg/mL (**Figure 1E** and **Table 1**). Simultaneous alterations in mitochondrial membrane functionality and plasma membrane integrity were observed only for aqueous extracts at concentrations equal to or higher than 7.06 x 10^{-3} mg/mL (**Figure 1E** and **Table 1)**. On the other hand, in assays with the ethanolic extract, dead PI^+ cells reached 7.90 ± 4.10 % in control cultures (diluent); however, in comparison with the aqueous extract, the ethanolic one induced a lower increase in the percentages of dead PI^+ cells (between 11 ± 0.10% and 24.14 ± 1.05%) at concentrations equal to or higher than 1.41 x 10^{-2} mg/mL (**Figure 1E** and **Table 1)**. In this case, simultaneous changes in mitochondrial membrane functionality and plasma membrane integrity were observed only for ethanolic extracts at concentrations equal to or higher than 5.65 x 10^{-2} mg/mL (**Figure 1E** and **Table 1)**.

After knowing the cytotoxic concentrations of both *L. operculata* extracts, sublethal doses were added to cultures of PBMCs from healthy controls to evaluate the kinetics of cell death by $DIOC_6$ staining every 24 hrs for 72 hrs. In control cultures (aqueous and ethanolic diluents), viability was above 95% at 0 hrs and decreased progressively to a minimum of 70% after 72 hrs. There were no differences in the kinetics of cell death between the plant extracts and their respective controls (diluents) at concentrations below 3.53 x 10^{-3} mg/mL. However, the $DIOC_6$ cell population decreased significantly after 72 hrs in cultures exposed to the aqueous plant extract at 7.06 x 10^{-3} mg/mL; at the same dose, the ethanolic preparation did not induce any change in mitochondrial membrane functionality (**Figure 2).**

After, to have some evidence of the type of cell death induced by the highest concentrations of *L. operculata* extracts, PBMCs from healthy volunteers were treated with high concentrations of the plant preparations for 72 hrs. Then, cells were labeled with FITC-conjugated-Annexin V/PI. Since double-positive Annexin V^+/PI^+ cells were not observed, PI^+ cells were excluded from the analysis. In comparison with their respective controls (diluents), only the aqueous extract at the highest concentration (5.65 x 10^{-2} mg/mL) induced the PS exposure in mononuclear cells **(Figure 3).** No changes were observed in cells treated with any concentration of the ethanolic plant extract (**Table 2**). In another set of similar experiments, cells were fixed with ethanol and stained with PI to evaluate ploidy. The relative DNA content was analyzed in terms of PI MFI; hypoploid cells were not observed in any of the cell cultures (**Figure 4).**

The previous results made it possible to set a range of non-cytotoxic concentrations of *L. operculata* extracts for the following assays; doses lower or equal to 3.53 x 10^{-3} mg/mL) were considered "safe" for cells. First, the PHA-induced proliferation of CFSE-labeled lymphocytes from healthy controls and patients with rhinosinusitis was evaluated by flow cytometry. After 96-h stimulation, lymphocytes were labeled with fluorescent anti-human CD3, CD4, and CD8 mAbs. In the presence of diluents, 30 - 40% of cells proliferated (CFSE low fluorescence); however, no significant differences were observed between $CD4^+$ and $CD8^+$ T-cells (**Figures 5C - F**) or between samples from healthy controls and patients (**Figures 5G - H**). Both plant extracts, at concentrations equal to or higher than 4.41 x 10^{-4} mg/mL, inhibited the PHA-induced proliferation of lymphocytes ($p < 0.01$). The $CD8^+$ T-cells were more sensitive to the ethanolic extract as the inhibitory effect was observed from a dose of 5.51 x 10^{-5} mg/mL. Nevertheless, there were no significant differences between healthy controls and patients with a history of rhinosinusitis.

It is important to note that the expression of CD69 showed a dose-dependent decrease in 12-h cultures, whether PHA-stimulation occurs before or after treatment with either of the plant extracts **(Figure 6).**

Supernatants from cultures of PHA-stimulated lymphocytes were collected to quantify cytokines. Results were similar for both plant extracts and between healthy controls and patients with a history of chronic rhinosinusitis. The levels of IL-2, IL-4, IL-8, TNF-α, and IL-12 decreased significantly in the presence of either plant extract in a dose-dependent manner; even the lower concentrations significantly reduced the accumulation of those molecules **(Figure 7).** In contrast, the concentrations of IL-1β, IL-10, and IL-6 showed a dual behavior: increased levels of IL-10 and IL-1β/IL-6 in the presence of low concentrations of plant extracts (1.37 x 10^{-5} mg/mL and 5.51 x 10^{-5} mg/mL, respectively); and a dose-dependent decrease of those cytokines in cultures exposed to the higher concentrations of the plant preparations.

Finally, the effect of *L. operculata* extracts on the PMA-induced respiratory burst of neutrophils and monocytes was evaluated (**Figure 8)**. The protocol used was an adaptation of the original one (Vowells et al., 1995), because the plant extracts quenched the rhodamine 123. Neither diluents nor *L. operculata* extracts had any effect on the respiratory burst of PMA non-activated phagocytes **(Figures 8C and F).** Both plant extracts, aqueous and ethanolic, at the highest concentrations (1.76 x 10^{-3} mg/mL and 3.53 x 10^{-3} mg/mL) inhibited strongly (nearly 100%) the PMA-activated respiratory burst of monocytes ($p < 0.05$) (**Figure 8G)**. Similarly, the PMA-activated respiratory burst of neutrophils was blocked by both extracts, but the percentage of inhibition was higher in the presence of the aqueous extract (90%) than the ethanolic one (50%) (**Figure 8D).** There were no differences between the results of healthy controls and patients with rhinosinusitis **(Figures 8C**- G).

4. Discussion

The current literature describes the immunomodulatory effects of non-toxic concentrations of compounds obtained from *L. operculata*, especially in cell and animal models. Some authors (Menon-Miyake et al., 2005; Silva et al., 2016) have approached the study of the *in vivo* effects of *L. operculata* preparations, reporting their effectiveness, but also emphasizing some adverse reactions, such as damage to the nasal mucosa, or toxicity at doses greater than 0.5 g/L, as shown in the present work. In the present study, a wide concentration range of *L. operculata* extracts was evaluated to discriminate cytotoxic from non-cytotoxic effects on human PBMCs.

Luffa operculata extracts at concentrations lower than 4.41 x 10^{-4} mg/mL do not affect the viability of human mononuclear cells.

The highest concentrations of aqueous and ethanolic extracts of *L. operculata* affected the viability of human mononuclear cells in a dose-dependent manner. Both plant extracts at doses greater than 1.41 x 10^{-2} mg/mL disrupted the mitochondrial membrane function and the cell membrane integrity; these changes were evidenced in the decreased mitochondrial transmembrane potential, the higher PS exposure on the outer surface of the plasma membrane, and the PI entrance and staining of DNA. The present results agree with a previous study that showed the high genotoxicity of the aqueous *L. operculata* extract at concentrations between 5 x 10^{-2} and 10 x 10^{-2} mg/mL (Baldissera et al., 2013); these doses induced a reduction in the number of cells in metaphase and augmented the breaks in the chromosomes of mononuclear cells (Ren et al., 2015). Although there were no differences in the ploidy of cells exposed to different doses of plant extracts in the present study, it is evident that *L. operculata* extracts can be cytotoxic depending on the concentration.

Different extracts of plants in the *Cucurbitaceae* family reduce mononuclear cells' viability in concentrations higher than 5 x 10^{-2} mg/mL. These high concentrations have been used to block the proliferation of tumor cell lines (A bdel-Salam et al., 2019; Baldissera et al., 2013; Lang et al., 2012; Sharma et al., 2015). *In vitro*, some compounds isolated from cucurbits, such as CuE, CuR and Dihydrocucurbitacin B, at concentrations lower than 5.5 x 10^{-4} mg/mL, exhibited anti-inflammatory effects due to the reduction of NO and NO2 and the inhibition of COX-2 (Abdelwahab et al., 2011; Escandell et al., 2007; Siqueira et al., 2007). *In vivo* murine models have demonstrated that low concentrations of cucurbit-derived compounds can reduce COX2, NOS2, and carrageenan-induced inflammation in plantar bearings (Peters et al., 2003 447; Siqueira et al., 2007 446). These findings reaffirmed the diversity of dose-dependent biological effects of cucurbit extracts and their derivatives.

***L. operculata* extracts inhibit the proliferation of human $CD4^+$ and $CD8^+$ T-cells**

The effect of *L. operculata* extracts on cell proliferation was evaluated on PHA-stimulated lymphocytes from healthy controls and patients with a history of rhinosinusitis. The plant extracts (aqueous or ethanolic) induced a reduction of 20% and 30% in the proliferation of $CD4^+$ and $CD8^+$ T-cells, respectively, in PHA-stimulated cultures. Results were similar for healthy controls and patients. It should be noted that lymphocytes were more sensitive to the ethanolic extract than to the aqueous one, but the differences between the intrinsic composition of the plant preparations are unknown. In a murine model, non-toxic concentrations of cucurbitacin IIB (a root-derived extract of the cucurbit *Hemsleya amabilis)* induced the accumulation in the G2/M phase of ConA-stimulated mononuclear cells. The effect was attributed to the diminished expression of cyclins B1 and D1, negative regulators of cell proliferation (Wang et al., 2016). A similar accumulation of cells in the G2/M phase is described in Jurkat T cells exposed to 5 x 10^{-3} mg/mL CuB; these cells are considered as more susceptible to the CuB effects for being isolated from a patient with leukemia (Zhu et al., 2012). Additionally, in studies of Jurkat cells exposed to CuE, the accumulation in the G2/M phase associated with phosphorylation of the eIF2 protein (Li et al., 2010). These results would explain the antiproliferative effect of *L. operculata* on $CD4^+$ and $CD8^+$ T-cells observed in the present study.

Other authors reported that non-toxic concentrations of CuD and CuB inhibited the proliferation of lymphocytes, as evidenced by the reduced thymidine incorporation into DNA (H. F. Smit, 2000; Nakanishi et al., 2016b; Whitehouse, 1969). Furthermore, CuD promoted cell death and autophagy in human T-cell leukemia lines (N akanishi et al., 2016a, b); and CuE suppressed the proliferation of alloreactive $CD4^+$ T-cells in human and murine models (Kim et al., 2018). It should be noted that triterpenes, especially cucurbitacins, can inhibit cell proliferation and induce cell death by blocking signaling pathways such as those mediated by JAK2/STAT3 (Boykin et al., 2011; Chan et al., 2010; Dong et al., 2010; M urtaza et al., 2017; T hoennissen et al., 2009; van Kester et al., 2008; Xie et al., 2016; Yar Saglam et al., 2016; Zheng et al., 2014) and MAPK (Chan et al., 2010; N. Baurin, 2002; Wang et al., 2014; Zheng et al., 2014). It would be interesting to study if these mechanisms also explain the antiproliferative effects of *L. operculata* observed in the present model.

***L. operculata* extracts prevent and inhibit PHA-induced activation of mononuclear cells.**

The early activation of mononuclear cells is evidenced through the surface expression of CD69 (Ziegler et al., 1994). In this study, the PHA-mediated activation of lymphocytes was evaluated before and after adding aqueous and ethanolic extracts of *L. operculate.* Both plant extracts inhibited the CD69 expression in a dose-dependent manner (even at the lowest concentrations), and even when added 2 hrs after the PHA stimulus. Other studies indicated that non-cytotoxic concentrations of CuIIb or CuB inhibit the CD69 expression on $CD3^+$ T-cells, and suggested that cucurbitacins might be the compounds responsible (Lueangamornnara, 2010; Wang et al., 2014). The preventing/inhibitory effects of *L.operculata* on T-cell activation, make these plant extracts promising antagonists of leukocyte activation even after this has already been triggered.

***L. operculate* extracts modulate the levels of cytokines in cultures of PHA-activated mononuclear cells**

PHA-stimulated lymphocytes, from healthy controls and

patients, were exposed to aqueous and ethanolic extracts of *L. operculata* to evaluate the levels of cytokines in culture supernatants. Both plant extracts caused a dose-dependent decrease in the accumulation of TNF-α, IL-4, IL-8, and IL-2. However, low concentrations of either extract induced increased levels of IL-10, IL-1β, and, to a lesser extent, IL-6, suggesting a possible dual immunomodulatory effect of the plant preparations. Previous studies have shown that cucurbitacins, especially CuB and CuE, inhibit the accumulation of TNFα, IL-2, IL-1β, IL-6, and IL-12 in cultures of LPS-stimulated murine macrophages and Jurkat cells stimulated with phorbol-12,13-dibutyrate and ionomycin. The authors of these investigations proposed the inhibitory effect on both cell types could be due to greater activation of the MAPK signaling pathways (Erk1/2, Jnk, HO-1), the concomitant lower activation of NF-κB, and the consequent inhibition of COX-2 (Kim et al., 2015; Qiao et al., 2013; Wang et al., 2015). According to these data, it is possible to suggest that those signaling pathways could also mediate the effects of *L. operculata* extracts observed in the present study. Further studies are needed to test this hypothesis.

Some studies have shown that CuE reduces the accumulation of IL-6, IL8, IL-1β, TNF-α in cultures of human bronchial epithelial and synoviocyte MH7A cells (Jia et al., 2015; Shang et al., 2019); whereas CuB reduces the levels of IL-10 in cells derived from myeloma, and CuI the levels of IL-6 in the human lung epithelial A549 cells (Guo et al., 2018; Yang et al., 2017). These results are opposite to those in the present study regarding the accumulation of IL-β and IL-10. However, in addition to the different cellular origins, the contradictory results could be attributed to different proportions of components in the *L. operculata* extracts. It is possible that the differential cytokine modulation is linked to components other than cucurbitacins, or that some compounds have dual activity. It is imperative to fractionate the extracts to evaluate the components that could be responsible for different activities.

***L. operculata* extracts inhibit the PMA-induced respiratory burst of human phagocytic cells**

PMA-activated monocytes and neutrophils were treated with *L. operculate* extracts to evaluate the respiratory burst. Production of ROS decreased in the presence of either plan extract at doses above $4.41x10^{-4}$ mg/mL; at a concentration of 3.53 $x10^{-2}$ mg/mL, the percentage of inhibition reached 100% and 80% in monocytes and neutrophils, respectively. Previous studies had shown that aqueous extracts (10 $x10^{-2}$ mg/mL) of *L. operculata* interacted with free radicals in mononuclear cells (Baldissera et al., 2013 543). In contrast, in the present study, considerably lower concentrations of aqueous extracts of *L. operculata* inhibited the respiratory burst, probably because of the different components of different extracts.

Some studies showed that increasing concentrations of extracts from plants of the *Cucurbitaceae* family reduced the PMA-induced production of ROS in human neutrophils (Fatma et al., 2015; Ielciu et al., 2019). Similarly, non-cytotoxic concentrations of cucurbitacins lowered the oxidative stress (ROS) in mouse macrophages stimulated with LPS and PHA (Cabral et al., 2020; Kim et al., 2015). Extracts from plants of the *Luffa* genus also diminished the production of ROS in the inflammation model in Wistar rats (Kumar et al., 2000; ULAGANATHAN IYYAMPERUMAL*, 2013). Moreover, several studies in cancer also corroborate the inhibitory effects of CuB mediated by reduction of ROS levels (Lin et al., 2019;

Luo et al., 2018; Ma et al., 2019; Ren et al., 2015; Yasuda et al., 2010; Zhang et al., 2012). These data suggest that cucurbitacins, present in all the preparations previously used, are the compounds responsible for the reduced cell production of ROS in different studies including the present one.

In conclusion, in the present study, low doses of aqueous and ethanolic extracts of *L. operculata* did not have cytotoxic effects on human mononuclear cells. These low doses did not alter either the PHA-induced activation or proliferation of lymphocytes. Likewise, these sublethal doses of the plant extracts downregulated the accumulation of TNF-α, IL-4, IL-8, IL-2, and IL-12, while a dual dose-dependent effect was observed for IL1-β, IL-10, and to a lesser extent for IL-6. On the other hand, high doses of the plant extracts inhibited the respiratory burst in phagocytes. The present data suggest a possible explanation for the anti-inflammatory effects previously attributed to *L. operculata* extracts. The present results are encouraging not only for the treatment of rhinosinusitis but also for a wide variety of inflammatory conditions, especially for being concentrations that do not compromise cell viability. Future studies would characterize the components present in these plant extracts and the particular combinations of compounds that could be used to minimize the adverse effects reported in treatments with *L.operculata* derivatives.

Conclusion: In a considerably lower range than toxicity can be evidenced, results pointed *L. operculata* as strong negative regulator of the proliferation and synthesis of proinflammatory cytokines by human lymphocytes, and the respiratory burst of phagocytes.

Declarations

Ethics: Patients and controls received appropriate information and signed the consent form approved by the Bioethics Committee of the Faculty of Medicine of the Universidad de Antioquia. A physician validated all clinical information. Individuals who had clinical symptoms or were taking any anti-inflammatory or antihistamine medication were excluded from the study.

Consent for publication

All authors consent all the described results here for publication.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Authors confirm that PRISM 5.0 data and FCS3.0 files (raw flow cytometry data) are available for detailed review of this manuscript.

Competing interests
The authors declare having no financial and non-financial conflict of interest with the publication of this article.

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Authors' Contributions
María Teresita Coneo Amaya and Ana María Daza Zapata, preformed the experiments. Jaime Iván Rodríguez O proposed the Project and was the grand recipient. He also contributed with the experimental design, data analysis and manuscript preparation. Mauricio Rojas-Lopez supervise all the flow cytometry procedures and cultures, participate in the manuscript preparation.

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Table and Figure legends

L. operculata (mg/mL)	$DIOC_6^{High}$ population (MFI)				PI^+ cells (%) Aqueous			
	Aqueous		Ethanolic		Aqueous		Ethanolic	
	X	± 95% CI	X	± 95% CI	X	± 95% CI	X	± 95% CI
0	12221	666	11846	219	6.47	1.54	7.90	4.10
6.89×10^{-6}	11869	211	11817	272	5.25	2.15	4.76	0.66
1.37×10^{-5}	12242	214	11725	218	5.43	1.97	4.58	1.48
2.75×10^{-5}	12221	432	11760	63	4.86	1.55	4.52	1.32
5.51×10^{-5}	11970	85	11995	156	6.59	0.01	6.74	0.14
1.10×10^{-4}	12415	44	12092	296	5.27	1.53	8.35	2.45
2.20×10^{-4}	12152	165	11990	355	5.60	1.70	4.56	1.16
4.41×10^{-4}	11818	111	11789	61	*10.70	3.70	8.70	1.30
8.82×10^{-4}	11750	166	12092	347	*11.15	3.45	7.65	3.05
1.76×10^{-3}	12512	798	12003	252	*11.10	3.00	8.91	0.20
3.53×10^{-3}	11929	36	11946	21	*11.08	3.63	9.35	0.26
7.06×10^{-3}	*11800	76	11939	16	*12.33	2.58	8.22	1.29
1.41×10^{-2}	**10378	48	11860	62	**22.35	8.95	*11.00	0.10
2.82×10^{-2}	**8923	740	11896	249	**43.33	11.02	*14.00	2.20
5.65×10^{-2}	***4581	373	*8495	91	**59.17	13.61	*17.65	2.75
1.13×10^{-1}	***4179	167	*8851	25	***97.15	2.55	**20.30	1.70
2.26×10^{-1}	***2333	314	*3637	963	***95.40	1.20	**24.15	1.05

Table 1. Effect of *L. operculata* extracts on the viability of human mononuclear cells Data from healthy volunteers from 24-h cultures. Asterisks indicate significant differences between cells treated with the plant extract and the respective diluent. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$; ANOVA I and Bonferroni post-test for mean contrast (n = 3).

Table 2: Phosphatidylserine exposure on mononuclear cells treated with cytotoxic concentrations of *L. operculata* extracts

FITC Annexin-V (MIF)

L. operculata (mg/mL)	Aqueous		Ethanolic	
	X	± 95% CI	X	± 95% CI
0	110	5	110	5
1.41×10^{-2}	114	4	114	4
2.82×10^{-2}	170	20	110	5
5.65×10^{-2}	**493	22	105	5

Data from PBMCs from healthy controls treated with plant extracts for 72 hrs.
** $p = 0.0012$ between cells treated with the plant extract and the respective diluent; ANOVA I and Bonferroni post-test for the contrast of means (n = 3).

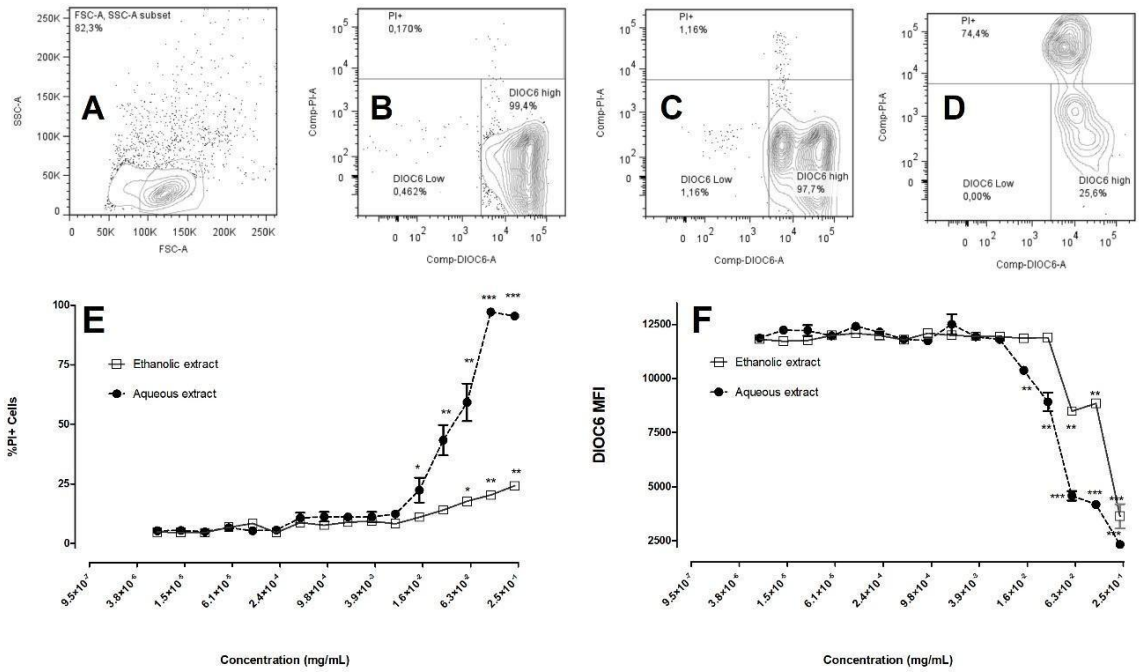


Figure 1: Effect of *L. operculata* extracts on the viability of human mononuclear cells. PBMCs from healthy volunteers were treated with *L.operculata* extracts at different concentrations for 0 – 72 hrs; then, cells were stained with 700 nM $DIOC_6$ and 1 µg/mL PI and acquired on the flow cytometer. Contour plot for cell gating according to size (FSC-A) and complexity (SSC-A) **(A)**; PI *vs.* $DIOC_6$ contour plots for cultures with ethanolic diluent **(B)**; ethanolic **(C)** and aqueous (**D)** extracts of *L.operculata* (both at 5.65 x 10^{-2} mg/mL)**.** Percentages of dead PI^+ cells **(E)** and MIF of $DIOC_6$ cell populations **(F)** in cultures exposed to different concentrations of the plant extracts. Mean and 95% CI are shown. Asterisks indicate significant differences between cells treated with the plant extract and the respective diluent: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ANOVA I and Bonferroni post-test for mean contrast (n = 3). (A - F Representative graphs from 24-h cultures).

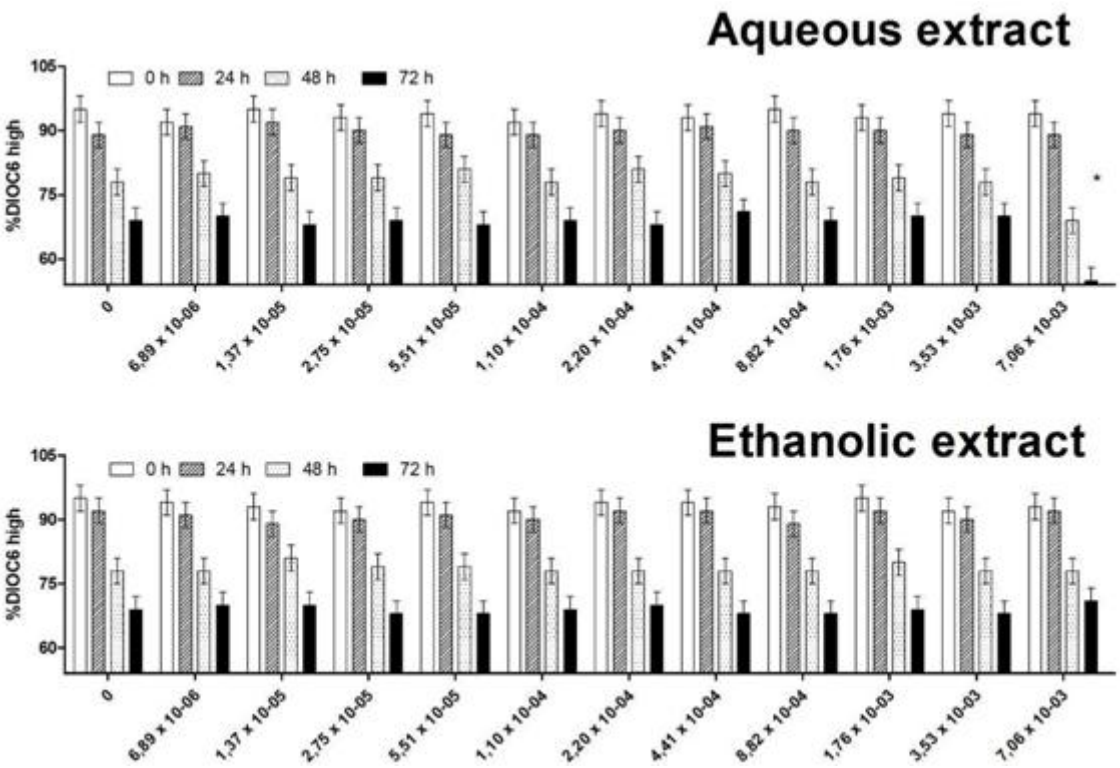


Figure 2: Kinetics of cell death in cultures of human mononuclear cells treated with *L. operculata* extracts. PBMCs were treated with sublethal concentrations (mg/mL) of the aqueous and ethanolic extracts of *L. operculata* for 0 - 72 hrs. Every 24 hrs, cells were stained with 700 nM $DIOC_6$/PI and acquired on the flow cytometer. The asterisk indicates significant differences between cells treated with the plant extract and the respective diluent: * $p < 0.05$; ANOVA I and

Bonferroni post-test for the contrast of means (n = 3).

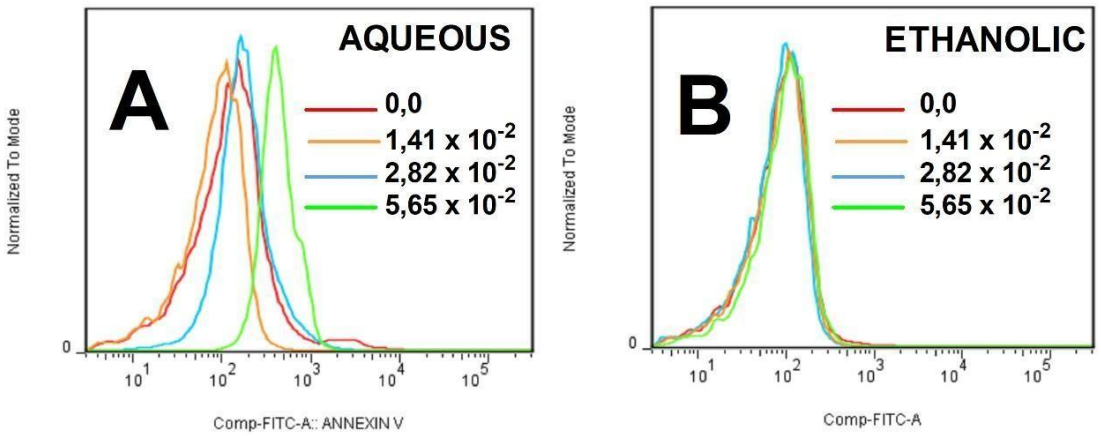
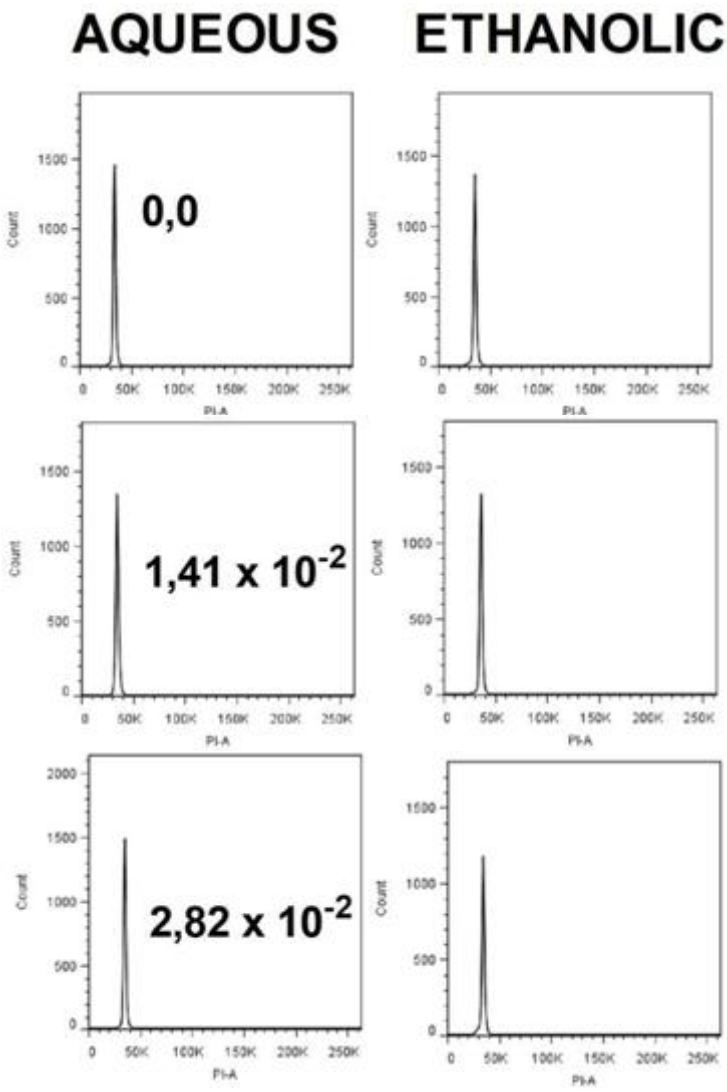


Figure 3: Phosphatidylserine exposure on mononuclear cells treated with cytotoxic concentrations of *L. operculata* extracts. PBMCs from healthy controls were cultured with aqueous and ethanolic extracts of *L. operculata* at high concentrations (mg/mL) for 72 hrs. Then, cells were stained with Annexin V^+/PI^+ and acquired on the flow cytometer. Since double-positive Annexin V^+/PI^+ cells were not observed, PI^+ cells were excluded from the analyses, and Annexin-V MFI data were registered (n = 3).



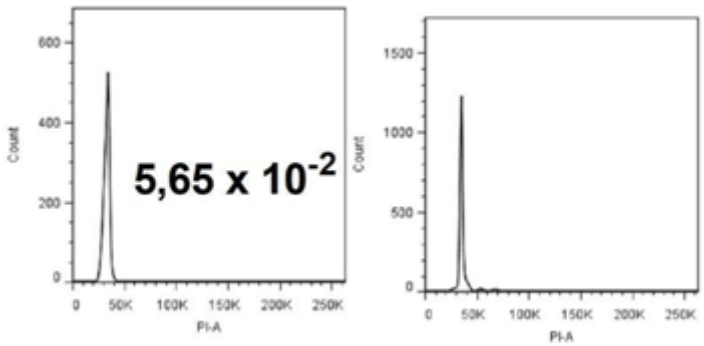


Figure 4: The relative content of DNA in human mononuclear cells treated with cytotoxic concentrations of *L. operculata* extracts

PBMCs were treated for 72 h with aqueous and ethanolic extracts of *L. operculata* at cytotoxic concentrations (mg/mL). Cells were then fixed with ethanol for 12 hrs, washed, stained with PI/RNase A, and acquired on the flow cytometer. Hypoploid cells were not observed (n = 4).

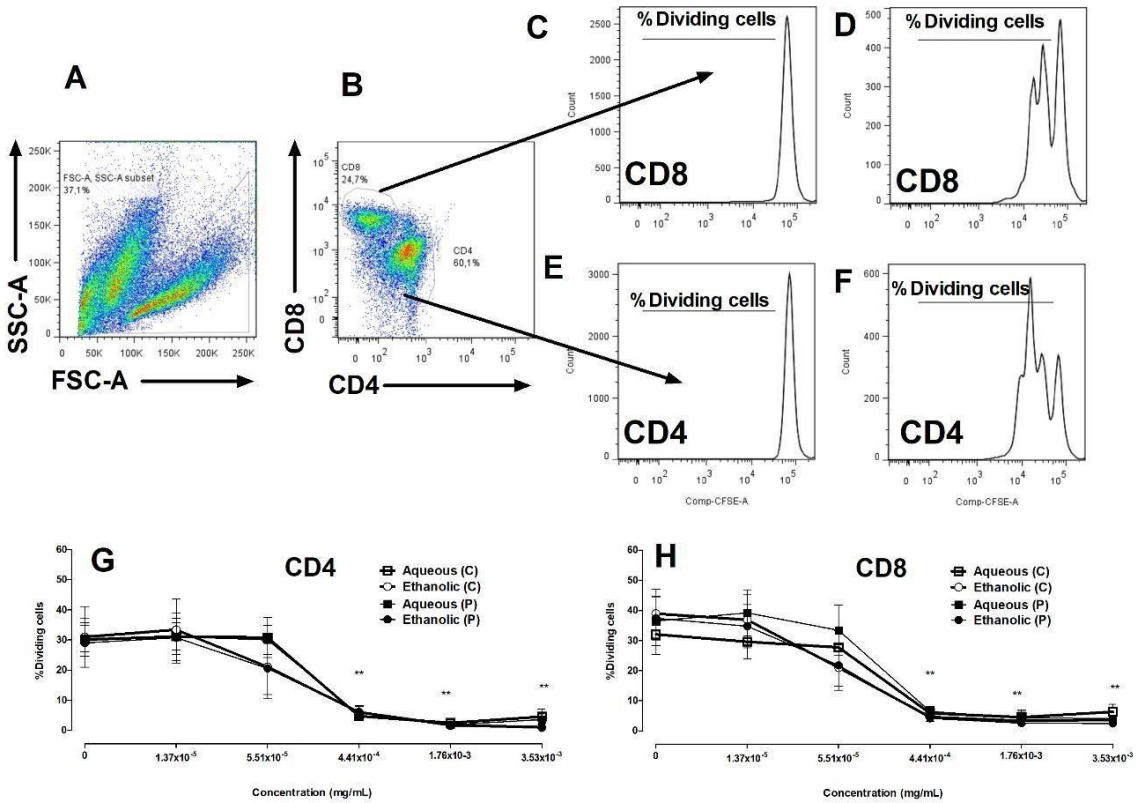


Figure 5: Effect of *L. operculata* extracts on PHA-induced proliferation of human lymphocytes.

PBMCs from healthy controls and patients with rhinosinusitis were stimulated with PHA in the presence of different sublethal concentrations of the aqueous and ethanolic extracts of *L. operculata* for 96 hrs. Dot plot for cell gating according to size (FSC-A) and complexity (SSC-A)-A) PI^+ cells were excluded from the analysis (not shown) **(A).** In the region of viable cells, $CD4^+$ and $CD8^+$ T-cells were gated **(B)** to analyze the CFSE dilution in $CD8^+$ T-cells **(C - D)** and $CD4^+$ T-cells **(E - F).** CFSE dilution in unstimulated **(C/E)** and PHA-stimulated cultures **(D/F).** Estimated percentages of proliferating $CD4^+$ **(G)** and $CD8^+$ **(H)** T-cells from cultures exposed to different concentrations of the plant extracts. Asterisks indicate significant differences between cells treated with the plant extracts and their respective diluents: * $p < 0.05$, ** $p < 0.01$; ANOVA II and Bonferroni post-test for the contrast of means (n = 9). Inside figures **(C)** healthy controls**, (P)** patients. (A - F representative graphs from a healthy control).

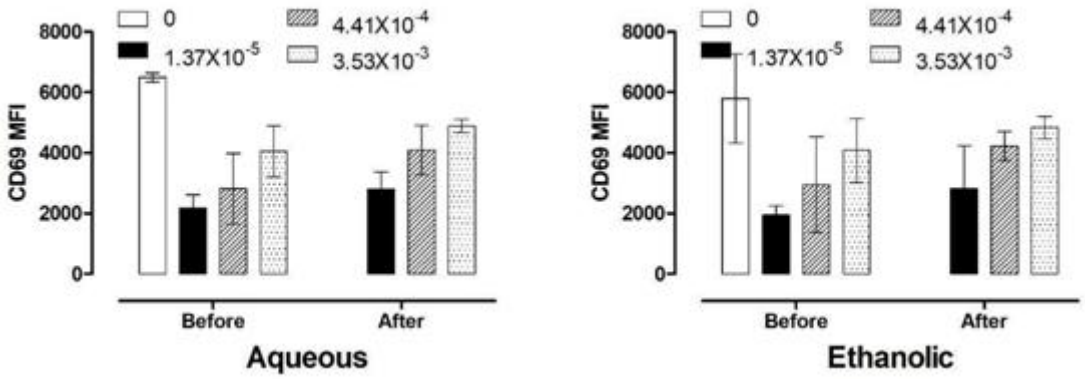


Figure 6: Early activation of PHA-stimulated human mononuclear cells treated with *L. operculata* extracts

PBMCs from healthy controls were cultured for 12 hrs with aqueous or ethanolic extracts of *L. operculata* before or after adding a PHA-stimulus. Cells were then stained with fluorochrome-conjugated mouse anti-human CD69/ECD and CD3/FITC mAbs and acquired in the flow cytometer. ANOVA II and Bonferroni post-test for the contrast of means (n = 5).

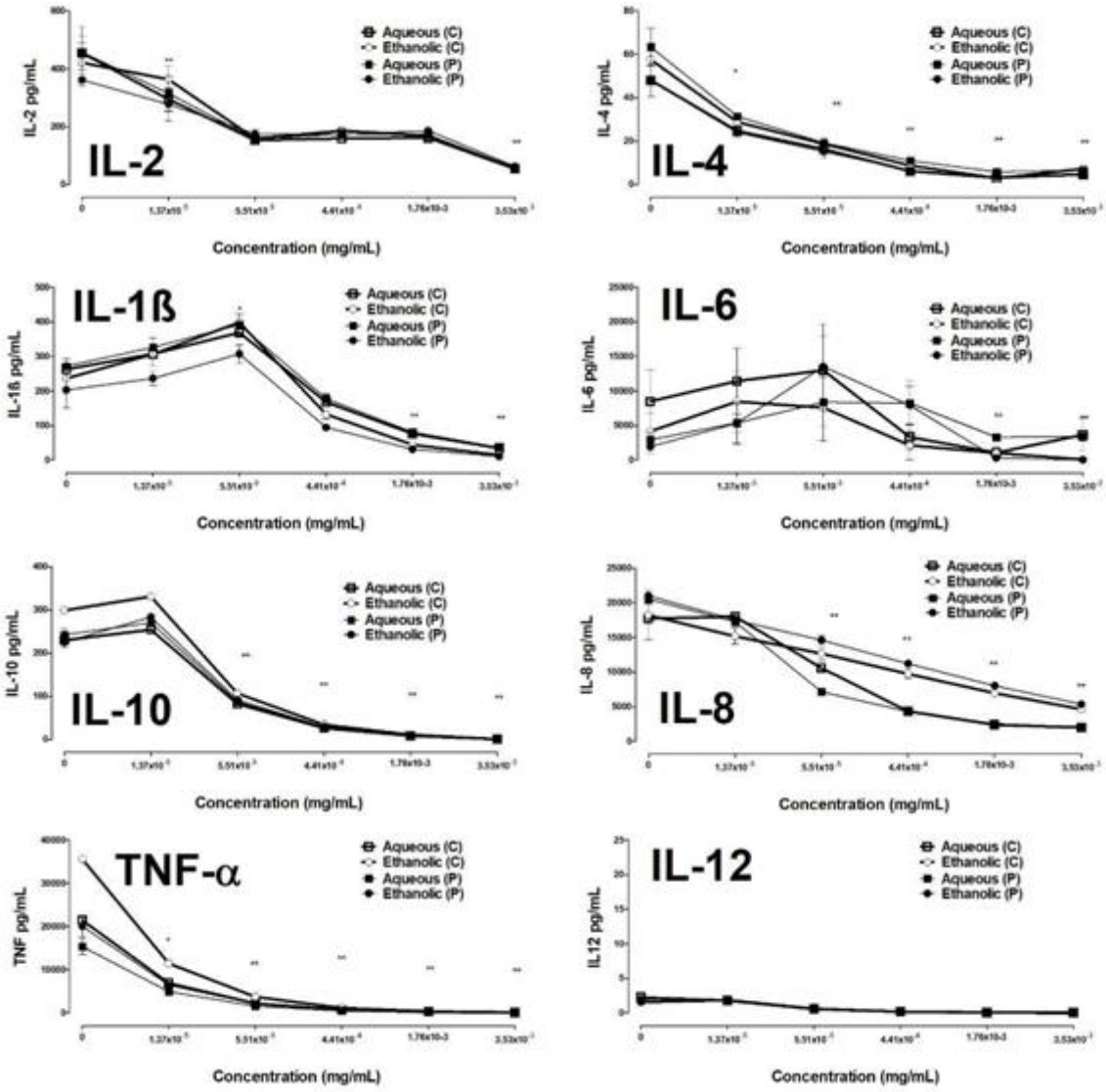


Figure 7: Levels of cytokines in cultures of PHA-activated human mononuclear cells treated with *L. operculata* extracts

PBMCs from healthy controls and patients with rhinosinusitis were stimulated with PHA in the presence of different sublethal concentrations of the aqueous and ethanolic extracts of *L. operculata* for 96 hrs. Cytokines were quantified by CBA and flow cytometry. Asterisks indicate significant differences between PHA-activated cells and PHA-activated cells exposed to plant extracts: $p < 0.05$ and ** $p < 0.01$; ANOVA II and Bonferroni post-test for mean contrast (n = 9). Inside figures **(C)** healthy controls, **(P)** patients.

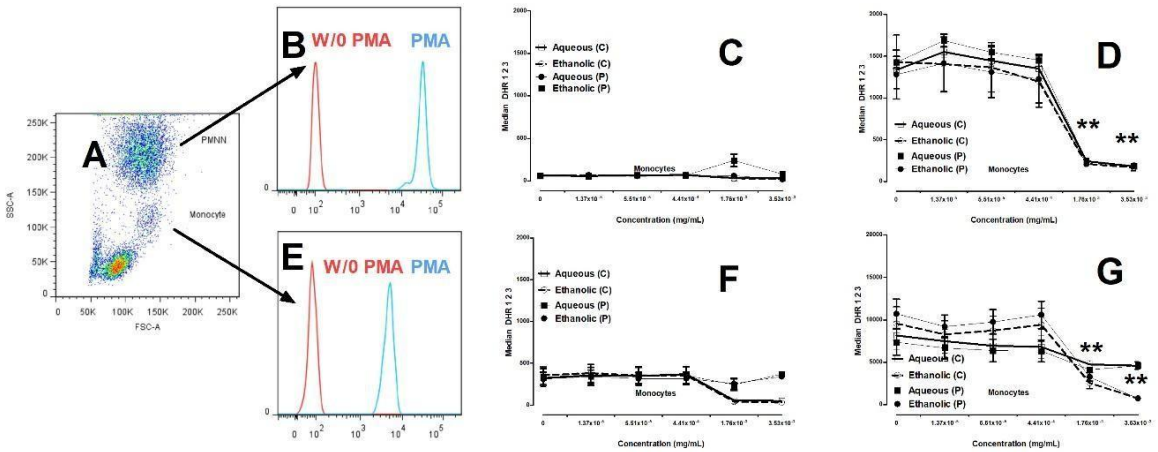


Figure 8: Respiratory burst of human phagocytes treated with *L. operculata* extracts

Samples of whole human blood from healthy controls and patients with rhinosinusitis were exposed to non-cytotoxic concentrations of the aqueous and ethanolic extracts of *L. operculata* for 30 min. Then, cells were incubated with DHR123 for 30 min and activated with PMA for 30 min. The oxidation of DHR123 to rhodamine 123 was evaluated by flow cytometry. Dot plots for gating neutrophils and monocytes according to cell size (FSC-A) and complexity (SSC-A**) (A).** Data from neutrophils **(B-D)** and monocytes **(E-G)**. Histograms of rhodamine 123 (MIF) in neutrophils and monocytes, respectively **(B/E).** Data from non-activated **(C/F)** and PMA-activated **(D/G)** cells from healthy controls and patients. Asterisks indicate significant differences between cells treated with the plant extract and the respective diluent: * $p < 0.05$; ANOVA II and Bonferroni post-test for the contrast of means (n = 9). Inside figures **(C)** healthy controls, (P) patients. (**A/B/E** representative graphs from a healthy control).

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