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IMMUNOSUPPRESIVE EFFECTS OF THE METHANOLIC EXTRACT OF *CHRYSOPHYLLUM CAINITO* LEAVES ON MACROPHAGE FUNCTIONS

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Abstract

Background: The aim of this work was to evaluate the immunomodulatory effect of the methanol extract (MeOH) from *Chrysophyllum cainito* leaves on the MΦs functions.
Material and Methods: Peritoneal murine MΦs isolated from Balb/c mice were treated with the MeOH extract and stimulated with LPS. The effect on the phagocytosis was evaluated by flow cytometry assay. The nitric oxide (NO) and hydrogen peroxide (H_2O_2) production was measured by the Griess reagent and phenol red reaction, respectively. Levels of IL-6 and TNF-α was measured using an ELISA kit. Viability of MΦs and Vero cells was determined by the MTT method.
Results: The MeOH extract of *C. cainito* leaves inhibited significantly the phagocytosis, and decreased IL-6 and TNF-α production as well as NO and H_2O_2 released by the MΦs, in a concentration-dependent manner. In addition, MeOH extract of *C. cainito* showed low cytotoxicity effect against the cells.
Conclusion: These results suggest that MeOH extract of *C. cainito* leaves has an immunosuppressive effect on murine MΦs, without effects on cell viability. GC-MS chromatogram analysis of MeOH extract showed that lupeol acetate and alpha-amyrin acetate are the principal compounds.

Keywords: Macrophages, Immunomodulation, *Chrysophyllum cainito*, Sapotaceae, Phagocytosis.

Introduction

Macrophages (MΦs) are widely recognized as the major components of the inflammatory and immunological reactions typically seen in several chronic diseases, including rheumatoid arthritis, atherosclerosis and diabetes (Oishi and Manabe, 2016). During an inflammatory response, MΦs have three principal roles: phagocytosis, antigen-presentation and immunomodulation through release of pro-inflammatory cytokines like tumor necrosis factor a (TNF-α), interleukin 6 (IL-6), interleukin 1f3 (IL-1f3), and several inflammatory mediators like nitric oxide (NO) (MacMicking et al., 1997; Fujiwara & Kobayashi, 2005; Stow et al., 2009). Therefore, MΦs play a crucial role in the initiation, maintenance and resolution of inflammatory processes (Wynn et al., 2013). The inhibition of phagocytosis and the release of pro- inflammatory mediators are a potential strategy to control inflammation (Underhill and Ozinsky,2002). From ancient times, Mayan traditional medicine in Mexico has relied largely on the use of plants (Gubler, 2010), including *Chrysophyllum cainito* (Sapotaceae), commonly known as caimito or star apple. The fruit is used in treating hemorrhage or is cooked and used for fever (Orwa et al., 2009; Parker et al., 2010). The infusion of the leaves has been used to treat diabetes mellitus and articular rheumatism (Das et al., 2010). Some studies has determined the biological activity of *C. cainito*. N'guessan et al. (2009) determined that the aqueous extract of *C. cainito* leaves produces a hypoglycaemic effect in rabbits, mainly through alkaloids, sterols or triterpenes identified in the plant. In an acute oral toxicity study in rats, Shailajan and Gurjar (2014). demonstrated that the aqueous and ethanolic (EtOH) extract of *C. cainito* leaves did not have toxic effects. Finally, Meira et al. (2014) verified that the methanolic (MeOH) crude extract, fractions and two triterpenes obtained from the *C. cainito* leaves possess

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important anti-hypersensitive properties against inflammatory pain in mice. Actually, few studies have showed the pharmacological properties of *C. cainito*, including on the immune system. Therefore, this study was designed to evaluate the immunomodulatory activity of the MeOH extract from *C. cainito* leaves on the phagocytic activity and pro-inflammatory mediators release in LPS- activated MΦs.

Materials and Methods

Materials

Dimethyl Sulfoxide (DMSO), 3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT), Lipopolysaccharides from *Escherichia coli* (LPS *E. coli* 0111:B4), Trypan-blue Dye, Cisplatin (CDDP), Propidium Iodide (PI), Sodium Nitrite (NaNO2), Griess reagent, Dextrose, Phenol red and Type I Horse Radish Peroxidase (HRP) were purchased from Sigma Aldrich (St. Louis, MO,USA). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin–Streptomycin were purchased from Gibco-BRL (Invitrogen-Gibco-BRL, Grand Island,NY, USA.). ELISA assay kits for measuring mouse TNF-α and IL-6 were obtained from Peprotech (London, UK).

Plant material and preparation of extract

C. cainito leaves (collected in October 2013 from Mérida, Yucatán, México) was authenticated by Dr. Salvador Flores Guido of the Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán (Voucher number Flores 9573). Dried and powdered leaves of *C. cainito* (80 g) were treated with MeOH at room temperature for approximately one week. The extract was filtered and the solvent was then evaporated under reduced pressure to give MeOH crude extract. The extract was diluted in DMEM media and filtered through 0.45 µm nylon filters prior to bioassays or chromatographic analysis.

Animals

Male Balb/c mice (20 ± 5 g) were obtained from Centro de Investigaciones Regionales "Dr.Hideyo Noguchi" of the Universidad Autónoma de Yucatán and maintained under standard laboratory conditions: pathogen- and stress-free environment, temperature of 22 ± 2°C, 12 h light/dark cycle, special food and purified water *ad libitum*. The animals were maintained in accordance with the principles and guidelines of National Institutes of Health (NIH) Guide for Treatment and Care for Laboratory Animals and by Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999).

Isolation and treatment of peritoneal murine MΦs

The mice were sacrificed by the method of cervical dislocation. In a laminar flow hood, the abdomen of the animals were disinfected with alcohol (70%) and peritoneal exudates cells were isolated by lavages of the peritoneal cavity using 10 mL of cold Phosphate Buffered Saline (PBS) and poured in sterile plastic tubes. Cells were harvested by centrifugation at 1800 rpm and 4°C for 15 min. Cells were pooled, re-suspended in supplemented DMEM media with 10% FBS and 1% of penicillin-streptomycin. The number of MΦs was determined in a hemacytometer.
Viability of the cells was determinate by trypan blue dye exclusion and was typically found to be 95%. Between $1x10^4$ cells/mL were seeded into each well of a 96 well plate (Costar, Cambridge, MA) for cell viability assay, H_2O_2, NO, TNF-ct and IL-6 production, and $1x10^5$ cells/mL were placed in each well of a 24 well plate for phagocytic activity assay and incubated at 37ºC, 5% CO2 and 95% air atmosphere by 48 h (Zhang et al., 1994). After removing the non-adherent cells, MΦs were treated with MeOH extract of *C. cainito* leaves at final concentrations per well of 1, 10, 100 and 200 tg/mL dissolved in supplemented DMEM media with 0.1% DMSO to a final volume of 200 tL for 96 well plates, 500 tL for 24 well plates and incubated for 24 h. The activation of MΦs was performed adding LPS of *E. coli* at 1 tg/mL in supplemented DMEM media and incubated during 48 h.

Cell line

Vero cells (cells of kidney from green monkey) were obtained from the American Type Culture Collection (ATCC CCL-81, Rockville, Maryland, USA) and maintained in supplemented DMEM media with 10% (v/v) FBS and penicillin (100 U/mL) with streptomycin (100 mg/mL). This cell line was maintained at 37°C and 5% CO2 atmosphere with 95% humidity.

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Cell viability

The cell viability was carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, previously described by Mossman (1983). Peritoneal MΦs were treated with MeOH extract of *C. cainito* (1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL) and plated in a 96-wells culture dish in a concentration of $1x10^4$ cells/well in supplemented DMEM media as negative control C(-) or 100% DMSO as positive control C(+). Vero cells were treated with MeOH extract of *C. cainito* (75 tg/mL, 150 tg/mL and 300 tg/mL) and plated in a 96-wells culture dish in a concentration of $2.5x10^4$ cells/well in supplemented DMEM media as C(-) or CDDP (1 tg/mL) as C(+). The cells were incubated overnight and washed with supplemented DMEM media. Later, 20 tL of the MTT solution (5 mg/mL) were added with 180 tL of DMEM media, and cells incubated 4 h at 37°C and 5% CO2. Finally, the supernatants were removed and 100 tL of DMSO at 100% were added to each well, dissolving the formazan crystals produced by MΦs and Vero cells. The absorbance of the MTT formazan was determined at 490 nm using the iMark Microplate Reader (Bio-Rad, USA). The percentage (%) of cell viability was calculated using the following formula: % Viability = [(Abs treated cells / Abs negative control) x 100]

Phagocytic activity

The phagocytic activity assay was determined according to the protocol described by Alonso- Castro et al. (2012) with some modifications. The murine peritoneal MΦs were seeded in 24-well plates at $5x10^5$ cells/well and incubated overnight. The MeOH extract of *C. cainito* leaves was added at different concentrations per well (1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL) and incubated during 24 h (37ºC and 5% CO2). The MΦs were co-cultured with *Saccharomyces cerevisiae* yeasts labeled with PI (100 tg/mL), in a concentration of $5x10^6$ yeast/well and were incubated 90 min at 37ºC and 5% CO2. The non-ingested labeled yeasts were removed and 500 tL of separation buffer (BSA 0.5%, EDTA 6.29 mM) was added and incubated at 37ºC and 5% CO2 for 10 min. The cell suspension was transferred to 1.5 mL conic tube and centrifuged for 10 min (150 x *g*, 4°C). The supernatants were removed and the cell pellets re-suspended in a 1% formaldehyde solution. MΦs activated with LPS co-cultured with *S. cerevisiae* yeasts labeled with PI were used as C(+) and MΦs without treatment and co-cultured with *S. cerevisiae* yeasts labeled with PI were used as C(-). Finally, the percentages (%) of phagocytosis were measured by the cellular fluorescent intensity emitted from the PI using a Cell Lab Quanta SC flow cytometer (Beckman Coulter, USA).

NO production

NO produced by MΦs was measured according to Ono et al. (2003), using the Griess reaction. Briefly, 100 tL of Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride in H2O and 1% sulfanilamide in 5% H3PO4) was mixed with 100 µL of cell culture supernatants of MΦs treated with MeOH extract from *C. cainito* leaves in a 96-well plate. MΦs activated with LPS in supplemented DMEM media were used as C(+) and cells in supplemented DMEM media without treatment were used as C(-). The plate was incubated during 10 min at room temperatureprotected from light. The absorbance was measured at 490 nm on the iMark Microplate Reader (Bio-Rad, USA) and nitrite concentration was determined by comparison with a NaNO2 standard curve (0-50 µM).

H2O2

Release

$_{H2O2}$ release by MΦs was determined following the protocol of Pick and Mizel (1981). A volume of 100 tL of fresh phenol red solution (5.5 mM dextrose, 0.056 g phenol red and 8.5 U/mL Type I HRP in DPBS) was mixed with 100 µL of cell culture supernatants of MΦs treated with MeOH extract from *C. cainito* leaves in a 96-well plate. MΦs activated with LPS in supplemented DMEM media were used as C(+) and cells in supplemented DMEM media without treatment were used as C(-). The plate was incubated during 3 h in completely darkness at room temperature. The reaction was stopped adding 10 µL of NaOH 1 N solution. The absorbance was measured at 620 nm on the iMark Microplate Reader (Bio-Rad, USA) and the $_{H2O2}$ concentration was determined by comparison with a $_{H2O2}$ standard curve (0-50 µM).

Cytokines production

Enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer's manual (Peprotech, London, UK) to determine the IL-6 and TNF-ct concentration in the supernatants of MΦs activated and treated with the MeOH extract from *C. cainito* leaves. MΦs activated with LPS in supplemented DMEM media were used as C(+) and cells in supplemented DMEM media without treatment were used as C(-). Capture antibody was used at a concentration

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of 2 tg/mL for IL-6 or 1 tg/mL for TNF-ct in PBS. Serial dilutions of recombinant IL-6 (0-4000 pg/mL) or TNF-ct (0-2000 pg/mL) were used as standard curve. IL-6 present in the supernatants was detected with a biotinylated second antibody avidin-HRP conjugated (0.5 tg/mL), while avidin peroxidase (0.25 tg/mL) was used to detect the levels of TNF-ct. The absorbance was measured at 490 nm using an iMark Microplate Reader (Bio-Rad, USA) and the concentration of the cytokines (pg/mL) was determined by extrapolation of the absorbances in the standard curve.

GC-MS analysis

GC-MS analysis was carried out on a 6890N Agilent Technologies equipped with a HP-5 capillary column (30 m, 0.25 mm I.D., film thickness: 0.33 mm) and 5973N mass detector. The extract (1 tL) was injected automatically in splitless mode. Injector and detector temperatures were set at 280°C and 290°C respectively. The septum purge flow rate was 20 mL/min. The gas (He) flow rate through the column was 0.8 mL/min, the column temperature was initially 80°C then gradually increased 15°C/min and finally the temperature was held at 310°C for 15 min. For GC-MS detection an electron ionization system was used with an ionization energy of 70 eV.

Statistical analysis

All the experiments were done in triplicate and the results were expressed in mean ± SD. Data were analyzed by using one-way ANOVA with Dunnett *post hoc* tests and the level of $P \leq 0.05$ was used as criterion of statistical significance. All calculations were done using GraphPad Prism® V5.03 software (GraphPad Software Inc., California, United States of America).

Results

Effect of MeOH extract of *C. cainito* leaves over the cell viability

The cytotoxic effects of MeOH extract of *C. cainito* leaves on MΦs and Vero cells was evaluated using the MTT assay. The multiple concentrations of methanolic extract from *C. cainito* leaves were used and effective doses were calculated from dose-response curve. Results of the cytotoxicity evaluation against MΦs and Vero cells of the MeOH extract of *C. cainito* leaves are shown in Figure 1. Treatments with 1, 10, 100 and 200 tg/mL for MΦs (Figure 1a), and 75, 150 and 300 tg/mL for Vero cells (Figure 1b) do not affect the viability of those cells at the concentrations used. The median cellular cytotoxic concentration (CC50) values in Vero cells were >300 tg/mL. The mean of the percentages ± standard deviation (SD) of three independent experiments were compared with a control group or C (-) in supplemented DMEM media without treatment.

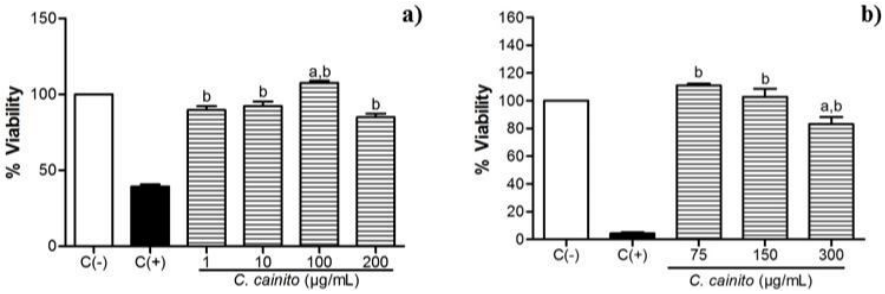

Figure 1: Effect of MeOH extract on viability of macrophages. Untreated macrophages were cultured in supplemented DMEM media as negative control or C(-). Macrophages were treated with DMSO 100% as positive control or C(+). b) Effect of MeOH extract on viability of Vero cells. Untreated Vero cells were cultured in supplemented DMEM edia as C(-). Vero cells treated with CDDP (1 tg/mL) were used as C(+). The percentages represent the mean ± SD of three independent experiments (n = 3). Letter "a" indicates significative differences in contrast to C(-) and "b" indicates significant differences in contrast to C(+), according to ANOVA test a dose dependent manner and reflected in the phagocytosis percentages 37.71%, 27.60%, 21.14% and 13.29% for 1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL respectively. The basal followed by Dunnett *post hoc* tests ($P < 0.05$). phagocytic activity found (5.43%) was treated as the negative control or C(-). MΦs stimulated with LPS (1tg/mL) were considered as the positive control C(+), with the highest percentage of phagocytic activity (65.20%).

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Effect of MeOH extract of *C. cainito* leaves on phagocytic activity by LPS-activated MΦs

The inhibitory effect of MeOH extract of *C. cainito* leaves on the phagocytic activity was determined by internalization of the *S. cerevisiae* yeast labelled with PI and measured using a flow cytometer (Figure 2). *C. cainito* treatments significantly suppressed phagocytosis activity in respectively (Figure 3a). The MΦs without LPS treatment produced 2.32 μM of nitrites after 24 h of incubation at 37ºC, corresponding to the C(-). In the same manner, the $_{H2O2}$ release by LPS-stimulated MΦs incubated with MeOH extract of *C. cainito* decreased in a concentration dependent manner to 9.66 μM, 8.08 μM, 6.90 μM and 6.38 tg/mL for 1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL, respectively (Figure 3b). LPS (1 tg/mL) in supplemented DMEM media was added to peritoneal MΦs and the $_{H2O2}$ release increased dramatically up to 24.80 μM, a value which was considered as the higher concentration of $_{H2O2}$ or C(+). MΦs without treatment produced 4.17 μM of $_{H2O2,}$ corresponding to the C(-).

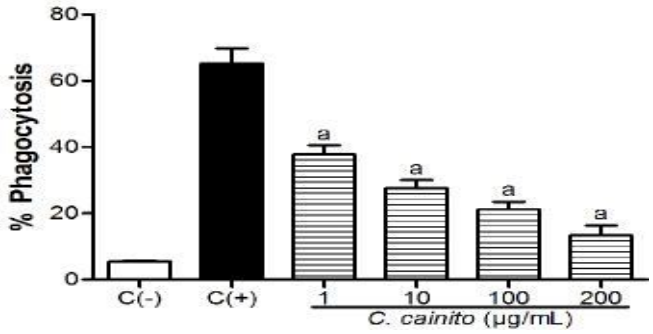

Figure 2: Effect of MeOH extract on phagocytosis activity of macrophages. Untreated macrophages were co-cultured in supplemented DMEM media and *S. cerevisiae* yeasts labelled with PI (1 tg/mL) as C(-). Macrophages were cultured in supplemented DMEM media with DMSO 0.1%, treated with LPS (1 tg/mL) and *S. cerevisiae* yeasts labelled with PI (1 tg/mL) as C(+). The percentages represent the mean ± SD of three independent experiments (n = 3). Letter "a" indicates significative differences in contrast to C(-), according to ANOVA test followed by Dunnett *post hoc* tests ($P < 0.05$).

Effect of MeOH extract of *C. cainito* leaves over NO and $_{H2O2}$ release

The exposure to LPS induced large amounts of nitrite in MΦs (21.77 tM), a value that was considered as the higher percentage of NO production and C(+). MeOH extract from leaves decreased the LPS-stimulated nitrite production in a concentration dependent manner to 19.61 μM, 17.40 μM, 14.43 μM and 13.11 μM at 1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL,

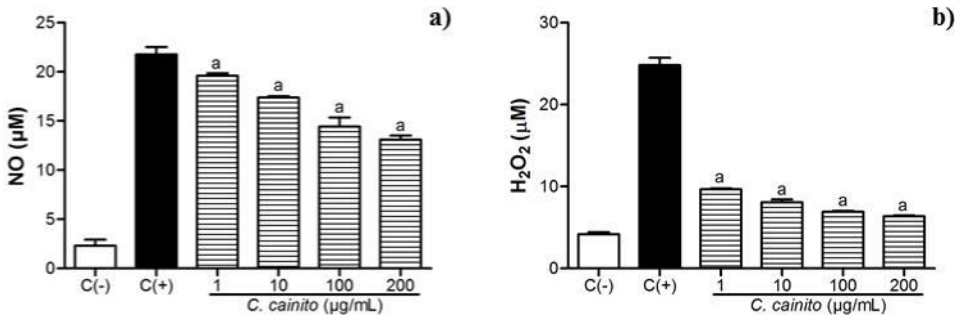

Figure 3: ffect of MeOH extract on NO production of macrophages. Untreated macrophages were cultured in supplemented DMEM media as C(-). Macrophages were cultured in supplemented DMEM media and treated

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with LPS (1 tg/mL) as C(+). b) Effect of MeOH extract on $_{H2O2}$ production of macrophages. Untreated macrophages were cultured in supplemented DMEM media as C(-). Macrophages were cultured in supplemented DMEM media and treated with LPS (1 tg/mL) as C(+). The percentages represent the mean ± SD of three independent experiments (n = 3). Letter "a" indicates significative differences in contrast to C(-), according to ANOVA test followed by Dunnett *post hoc* tests ($P <$ 0.05).

Effect of MeOH extract of *C. cainito* on MΦ-related cytokines production

To assess the effects of *C. cainito* on IL-6 and TNF-α production by activated MΦs, these cells were incubated with increasing concentrations of *C. cainito* and the quantities of the cytokines secreted into the culture supernatants were monitored by ELISA. MeOH extract of *C. cainito* leaves decreased the IL-6 and TNF-α, secretion in the supernatants, in a dose-dependent manner. The concentrations of IL-6 were 2072.00 pg/mL, 1812.00 pg/mL, 1339.00 pg/mL and 1075.00 pg/mL at 1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL, respectively (Figure 4a). In the case of TNF-α, the concentrations calculated were 865.70 pg/mL, 615.70 pg/mL, 495.70 pg/mL and 250.70 pg/mL for 1 tg/mL, 10 tg/mL, 100 tg/mL and 200 tg/mL, respectively (Figure 4b). The diminished levels of all the concentrations of this cytokines were statistically significant in contrast to its respectively negative (85.33 pg/mL and 150.70 pg/mL for IL-6 and TNF-α, respectively) and positive (3309.00 pg/mL and 1061.00 pg/mL for IL-6 and TNF-α, respectively) controls.

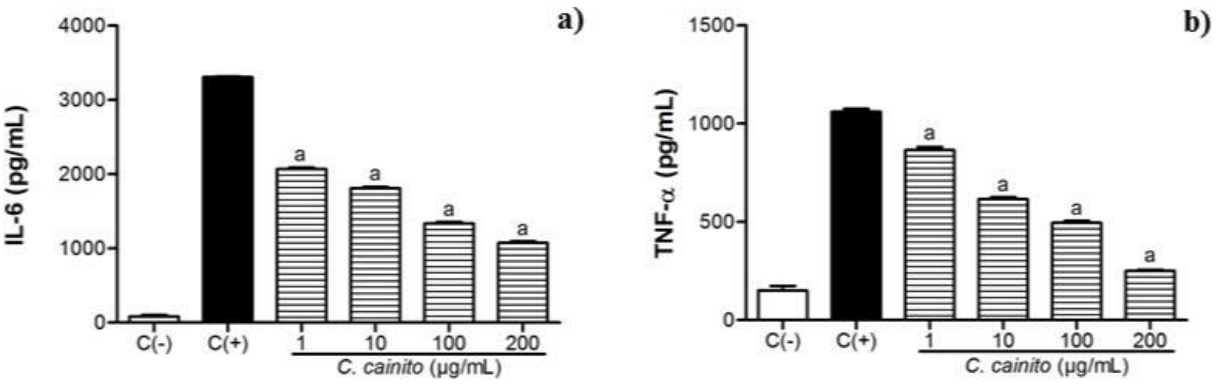

Figure 4: Effect of MeOH extract on IL-6 production of macrophages. Untreated macrophages were cultured in supplemented DMEM media as C(-). Macrophages were cultured in supplemented DMEM media and treated with LPS (1 tg/mL) as C(+). b) Effect of MeOH extract on TNF-a production of macrophages. Untreated macrophages were cultured in supplemented DMEM media as C(-). Macrophages were cultured in supplemented DMEM media and treated with LPS (1 µg/mL) as C(+). The percentages represent the mean ± SD of three independent experiments (n = 3). Letter "a" indicates significative differences in contrast to C(-), according to ANOVA test followed by Dunnett *post hoc* tests ($p < 0.05$).

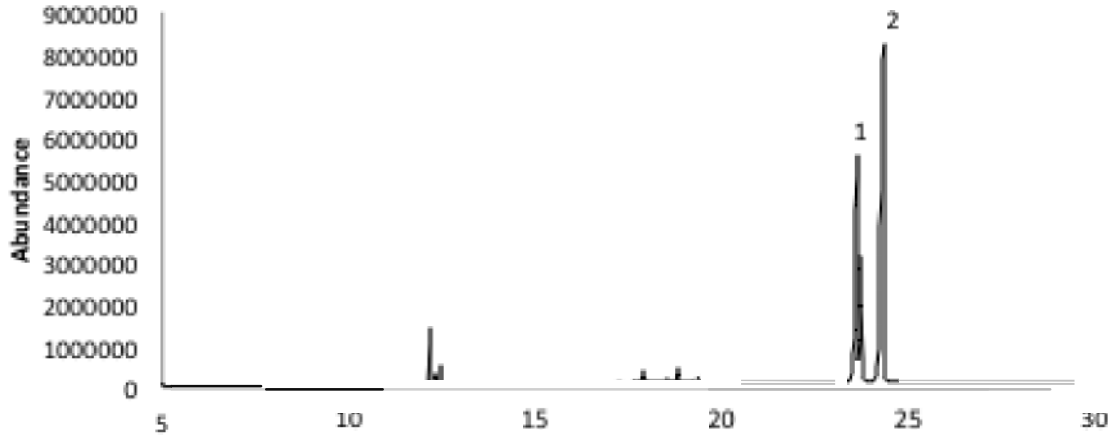

Figure 5.: GC chromatographic profile of MeOH extract from *C. cainito* leaves. (1) alpha-amyrin acetate and (2) lupeol acetate

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Chromatographic analysis

Analysis of the cromatogram obtained from the MeOH extract from *C. cainito* leaves showed the presence of two principal compounds. The identification of lupeol acetate and alpha-amyrin acetate were based on the mass spectrum and by comparison with mass spectra libraries (Database NIST02).

Discussions

Although the genus *Chrysophyllum* is distributed throughout the American continent and other parts of the world, very few works are found in the literature about the plants of this genus, including *C. cainito*. In this study, we observed the viability of MΦs remained intact during 24 h incubation period with *C. cainito* MeOH extract, indicating the inhibition obtained in phagocytic activity not to be the consequences of cytotoxicity of extract. Some studies have described that the crude extract, fractions and pure compounds (mainly triterpenes) obtained from the *C. cainito* leaves possess inflammatory and anti-hypersensitive properties in mice models (Meira et al 2014). The mechanisms through which *C. cainito* exerts its inflammatory actions are still unclear. In response to LPS-stimulation, MΦs produce pro-inflammatory mediators such as NO (Reddy and Reddanna, 2009). LPS-induced NO production is mediated by the inducible nitric oxide synthase (iNOS) expression. An excessive secretion of cytokines and iNOS expression mediated NO production (Jorens et al., 1995). Our results demonstrated that the MeOH extract of *C. ainito* leaves caused an inhibitory effect on phagocytic activity, as NO and $_{H2O2}$ production. Dupuy et al. (2013) reported that lupeol (triterpene present in *C. cainito* leaves according to Meira et al., 2014) exerted a decrease in the phagocytic activity and NO production of PBMC, which share the results obtained on the MΦs treated with the extract of *C. cainito*. Equally, Duarte et al. (2001) evaluated the action of 27 vegetable compounds and comprobated that lupeol decreases the level of $_{H2O2}$ in peritoneal murine MΦs. Schmid et al. (2009) reported that triterpenes inhibit NO production by reducing iNOS expression, without affecting activity of the enzyme. In the same way, Kim et al. (2009) explained that the anti-inflammatory activity of these compounds is associated to the decreased production of iNOS and cyclooxygenase-2 (COX-2). The cytokines are involved in virtually every facet of immunity and inflammation, including innate immunity and cellular activation (Borish and Steinke, 2003); for example, IL-6 and TNF-α help in acute phase response, and the TNF-α induces the expression of adhesion molecules, causing chemotaxis of leucocytes (Ikram et al., 2004). Our study showed that the MeOH extract of *C. cainito* leaves caused an inhibitory effect on cytokines production. The only study evaluated someone immune property of *C. cainito* has reported that the chloroform (CHCl3) fraction reduce the TNF-α levels in mice injected with carrageenan. In this fraction was found two triterpenes (lupeol acetate and lup-20(29)-en-3β-O-hexanoate), providing evidence that the anti- inflammatory of *C. cainito* is due, in part, to the presence of these compounds (Meira et al.,2014). Lucetti et al. (2010) showed that lupeol acetate decrease the tissue levels of TNF-α. Inclusive, Ashalatha et al. (2010) has showed that lupeol acetate presents an anti-inflammatory activity by regulating TNF-α and IL-2. Ding et al. (2009) identified beta-amyrin acetate and a mixture of beta-amyrin and lupeol isolated from *Rhus sylvestris* (triterpenes present in *C. cainito* leaves according to Lopez, 1983), and they concluded that the mixture reduce the IL-6 and TNF- α secretion in a concentration dependent manner; beta-amyrin acetate reduced the IL-6 and TNF- α secretion, too.

Conclusions

Then, this is the first study about the evaluation of the MeOH extract from *C. cainito* leaves on the immune system, showing an important immunosuppressive effect on the peritoneal murine MΦs, in a concentration dependent manner and without affect the viability of MΦs and Vero cells. *C. cainito* significantly inhibited the phagocytosis, IL-6 and TNF-α production and decreased the NO and $_{H2O2}$ release by the MΦs, in a concentration dependent manner. The principal metabolites presents in the *C. cainito* leaves were lupeol acetate and alpha-amyrin acetate, is possible that these molecules are implicated in the immunosuppressive effects. Although, more phytochemical and pharmacological studies are necessary.

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