Brazilian essential oil of *Cymbopogon martini* (Roxb.) Will. Watson (Poaceae): positive effects on inflammation-induced human fibroblasts and skin aging

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Abstract

The study evaluated the effects of essential oil from *Cymbopogon martini* (Roxb.) Will. Watson (CMEO) on lipopolysaccharide (LPS)-stimulated human fibroblasts. Samples were collected in Monte Verde, Minas Gerais, Brazil and analyzed by gas chromatography with mass spectrometry. The fibroblasts were cultured in a monolayer using Iscove's medium and stimulated by LPS (1 µg/mL) and incubated for 24 h at 37°C. The cytotoxicity was evaluated by MTT assay and collagen concentration by Sirius red. Collagenase activity, hyaluronic acid, and the concentrations of IL-1β; IL-6; MCP-1 (CCL2), and MIP-1-α (CCL3) were evaluated. The effect of CMEO on the expression of mRNA and the secretion of enzymes in fibroblasts were evaluated by RT-qPCR and ELISA, respectively. CMEO was cytotoxic against fibroblasts, in which 10 µg/mL inhibited 50% of cell viability. When treated with CMEO, the fibroblasts produced more collagen and hyaluronic acid than control cells. When stimulated by LPS, fibroblasts exhibited higher production of IL-6, IL-1β, MCP-1, and MIP-1α than control cells. The study demonstrated the effects of CMEO on the modulation of mediators related to inflammation and decreasing the mRNA and secretion levels of metalloproteinases, revealing to be a promising candidate for anti-aging effects and wound healing treatments.

Keywords: *Cymbopogon martini*. Anti-inflammatory. Anti-aging. Hyaluronic acid. Collagen.

Introduction

Essential oils (EOs) (Aetherolea, Ethereal oils, Volatile oils) are concentrated hydrophobic liquids containing volatile aromatic compounds from the secondary metabolism of plants[1,2]. EOs are principally composed of mono- and sesquiterpene hydrocarbons and their oxygenated derivatives, along with aliphatic alcohols, aldehydes, and esters[3]. The knowledge of EOs chemical profile is important to select the suitable extraction method[3].

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EOs can be extracted from plant tissues, such as flowers, fruits, leaves, peel, stem, and roots[1,2]. Although they are largely used in the pharmaceutical and food industry, it is unknown exactly whether EOs were used as healing agents or for domestic use at the beginning[3].

Cymbopogon martini (Roxb.) Will. Watson (CM), as known as palmarosa, is a member of the Gramineae family, which is very famous for its high oil content[1]. *Cymbopogon martini* (Roxb.) Will. Watson essential oil (CMEO) has been widely used in aromatherapy because of its antimicrobial and anti-inflammatory properties [4]. The bioactivity of EOs depends on the interaction of their compounds which results in a synergic or an antagonic effect[3]. Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene alcohol [4]. This monoterpenoid is the major compound of Palmarosa EO and has anti-oxidant and anti-inflammatory effects[4,5].

Fibroblasts are one of the most important cells involved in tissue repair because they produce a group of molecules to maintain the extracellular matrix (ECM) and replace wounded tissue, such as collagen type I, III, and IV, fibronectin, glycosaminoglycans, hyaluronic acid, laminins, metalloproteinases and proteoglycans[6,7]. In addition, ECM reorganization occurs through a process of degradation and crosslinking enzymes, produced by fibroblasts, that are activated and regulated by pro-inflammatory cytokines[7].

Although inflammation is a process well-regulated, an excessive inflammatory response can be a source of additional damage to individuals, such as inflammatory diseases[8]. Thus, the use of Palmarosa EOs as an anti-inflammatory agent can be an effective strategy to regulate an excessive inflammatory response[8]. The aim of the present study was to evaluate the effects of CMEO on lipopolysaccharide-stimulated human fibroblast cells.

Materials and Methods

Iscove's Modified Dulbecco's Medium (IMDM), fetal bovine serum (FBS), penicillin- streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco BRL (Grand Island, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Plant identification

Samples of *Cymbopogon martini* (Roxb.) Will. Watson were collected in the Monte Verde, State of Minas Gerais, Brazil, September 2019. An exsiccate of the plant collected and identified was deposited and authenticated (voucher specimen number HUMC 7661) in the Herbarium of the University of Mogi das Cruzes, SP, Brazil.

Cymbopogon martini (Roxb.) Will. Watson essential oil

The Essential oil of *Cymbopogon martini* (Roxb.) Will. Watson was supplied by WNF Essential Oils (São Paulo, SP, Brazil). The purity and quality of the *Cymbopogon martini* (Roxb.) Will. Watson essential oil were monitored by the WNF Essential Oils quality control department. *Cymbopogon martini* (Roxb.) Will. Watson chemical analysis was performed in the Analytical Center of the Chemistry Institute of the University of São Paulo (USP), by gas chromatography-mass spectrometer (GC-MS) Shimadzu model QP5050A, according to operating conditions: CBP-5 capillary column (50 m × 0.25 mm × 0.25 μm), injector temperature of 250°C

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and helium (He) as a carrier gas. The energy of impact used in MS was 70 eV. Compounds identification in EO was carried out by mass spectra analysis according to the National Institute of Standards and Technology library. The relative concentration of each compound in essential oil was computed from GC peak areas by the analysis program.

Culture and cytotoxicity evaluation of CMEO by MTT assay

The CCD1072Sk cell line was obtained from Rio de Janeiro Cell Bank (CCD1072Sk - ATCC CRL2088). The cells were cultured in a monolayer using IMDM (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 UI/mL penicillin/streptomycin, and 0.25 µg/mL Fungizone (Gibco) in a humidified atmosphere at 37°C in 5% CO_2. These cells were trypsinized three times per week using 0.25% trypsin/EDTA (Cultilab, Brazil). For the 24-hour cell viability assessment, the control and treated cells were centrifuged and resuspended in equal parts medium and trypan blue (0.05% solution) and counted using a hemocytometer. To evaluate the cytotoxicity of CMEO, the essential oil was dissolved in the culture medium in appropriate concentrations. The cell viability of control and CMEO (0.0–160.0 µg/mL)-treated fibroblasts cells were measured using a standard MTT assay. Briefly, 5×10^4 viable cells were seeded into clear 96-well flat-bottom plates (Corning) in IMDM medium supplemented with 10% fetal bovine serum (FBS) and incubated with different concentrations of the extract for 24h. Then, 10 µL/well of MTT (5 mg/mL) was added and the cells were incubated for 4h. Following incubation, 100 µL of 10% sodium dodecyl sulfate (SDS) solution in deionized water was added to each well and left overnight. The absorbance was measured at 595 nm using a FlexStation 3 Multi-Mode Benchtop Reader (Molecular Devices, Sunnyvale, CA, USA).

Cell viability after LPS treatment

Briefly, cells were seeded in 96-well culture plates at a density of 5×10^4 viable cells/well and incubated for 24 h, then exposed to IC50 concentration of CMEO (10 µg/mL), previously determined in item 2.1 in the presence of LPS (1 µg/mL) and incubated for another 24 h at 37°C. The MTT solution was added to a final concentration of 0.5 mg/mL and then incubated for 2 h at 37°C followed by the addition of 0.1 mL of dimethyl sulfoxide to dissolve the MTT-formazan. The amount of MTT-formazan was then determined by measuring abs at 595 nm.

Sirius Red Collagen Quantification

The cells were pre-treated in serum free medium for 6 hours and were then treated with 10 µg/mL (IC_{50} concentration) of CMEO for 24 hours. After cells were cultured, the medium was removed, and the wells were washed three times with 0.1 M PBS. Next, 100 µl of Bouin's solution (picric acid 0.9%, formaldehyde 9.0% and glacial acetic acid 5.0%) were added for fixation for 1 h. Samples were washed with PBS, then the Sirius Red dye was added. After 1 h, the maximum possible amount of dye was removed, followed by washing with 150 µl of a 0.01 M hydrochloric acid solution for 30 seconds to remove the dye that did not bind to collagen. Next, the dye was removed from cell layers by the addition of 0.1 M NaOH for 30 min. 100 µL aliquots of the solution contained in the wells were transferred to a new plate. Absorbance was measured with an Elx- 800-UV (Bio-Tek Instruments, USA) microplate reader at 570 nm.

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Hyaluronic acid synthesis assay

Effects of CMEO on HA synthesis were determined using an ELISA kit. The fibroblast cells (5.0×10^4 cells/well) were seeded in 96-well-plates for 24 h at 37°C, 5%CO_2. The cells were pre-treated in serum free medium for 6 hours and were then treated with 10 μg/mL (IC_{50} concentration) of CMEO for 48 hours. The cultured medium was collected, and HA synthesis was measured by ELISA. The absorbance was measured at 450 and 570 nm using a microplate reader. The HA concentration in the cultured supernatant acquired from the treated fibroblast cells was calculated and compared with the standard curve of HA.

Collagenase activity

The collagenase activity was analyzed by using enzyme-linked immunosorbent assay (ELISA) kits (ABCAM, USA) following the manufacturer's instructions. Briefly, the cells (5.0×10^4 cells/well) were seeded in 96-well-plates for 24 h at 37°C, 5%CO_2. Fibroblast cells were pre-treated in serum free medium for 24 hours and were further treated with CMEO (10 μg/mL) for 48 hours.

Cytokines and chemokines analysis in cell culture supernatants

The concentrations of IL-1β; IL-6; MCP-1 (CCL2); and MIP-1-α (CCL3) in the cell culture supernatants were analyzed by using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Cells were treated with LPS (1 μg/mL) with or without CMEO (10 μg/mL) for 6 h. The cell culture supernatant (100 μL) was collected to determinates the levels of cytokines and chemokines, according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR)

The effect of CMEO on the expression of mRNA of MMP-1; MMP-2; and MMP-9 enzymes in fibroblasts was evaluate by RT-qPCR. Cells were pretreated with CMEO (10 μg/mL) for 2 hours and treated with LPS (1 μg/mL) for 24 hours. Total RNA extracted from cells samples was converted to cDNA using a SuperScript® III RT kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The concentration of RNA was detected using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). GAPDH was used as the internal control. The thermocycling conditions were as follows: 95˚C for 10 min followed by 36 cycles of 94˚C for 15 sec and 57˚C for 40 sec. The $2^{-\Delta\Delta Cq}$ method was used to quantify the relative gene expression levels of the target genes. Relative standard curves were generated by serial dilutions and all samples were run in triplicates. Following, the sequence of primers used in the qRT-PCR analysis: MMP-1: Forward 5'-AGCTAGCTCAGGATGACATTGATG-3'; and reverse 5'-GCCGATGGGCTGGACAG-3'; MMP-2: Forward 5′-ACCGCGACAAGAAGTATGGC-3′; and Reverse 5′-CCACTTGCGGTCA TCATCGT-3′; MMP-9: Forward 5′-CGATGACGAGTTGTGGTCCC-3′; and Reverse 5′-TCGTAGTTG GCCGTGGTACT-3′; GAPDH: Forward 5′-CGGTGTGAACGGATTTGGC-3′; and Reverse 5′-GTGAGTGGAGTCATACTGGAAC-3′.

Enzyme-linked immunosorbent assay for the determination of MMP2 and MMP9

Cell supernatant was centrifuged at 12,000g for 15 min at 4°C. MMP2 ELISA kits (RAB0365; Sigma, USA) and MMP9 ELISA kits (RAB0372; Sigma, Nanjing, China) were used to measure the levels of protein secretion of MMP2 and MMP9 according to the manufacturer's instructions. To detect MMP-1 concentration

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in supernatants, ELISA was performed with a commercially available ELISA kit (Abcam). The MMP-1 concentrations were detected according to the manufacturer's instructions

Statistical analysis

The obtained results were expressed as the mean ± standard error of mean (SEM) from at least three independent experiments, unless stated otherwise. Paired data was evaluated by Student's t-test. A p value of <0.05 was considered significant.

Results and Discussion

The main chemical components obtained by gas chromatography are shown in **TABLE 1**.

TABLE 1: Chemical compositions of CMEO leaf oil.

RT*	Chemical composition	Area %
8.818	Geraniol	81.70
10.488	Geranyl acetate	10.21
6.525	Linalool	01.57
9.014	Geranial	01.23
11.185	Isocaryophyllene	01.09

* Retention Time

The effects of the CMEO on CCD1072Sk fibroblasts cell viability was evaluated using the MTT assay. As shown in **FIGURE 1A**, the CMEO was cytotoxic against fibroblasts cells, in which 10 µg/mL inhibited 50% of cell viability. The IC50% value obtained corroborates the variability found in the literature in studies where screenings are carried out with different types of fibroblasts and essential oils. CMEO was able to reverse the cytotoxicity caused by LPS in all these cell lines (**FIGURE 1B**).

FIGURE 1: (A) Cell viability of CMEO on fibroblast cells. The cells were treated with or without various concentrations of the each one (0.0–160.0 µg/mL) for 24 h. The cell viability was determined using MTT assay. (B) Effects of CMEO on LPS-induced cytotoxicit. All assays have been performed in triplicate and the mean ± standard deviations are shown.
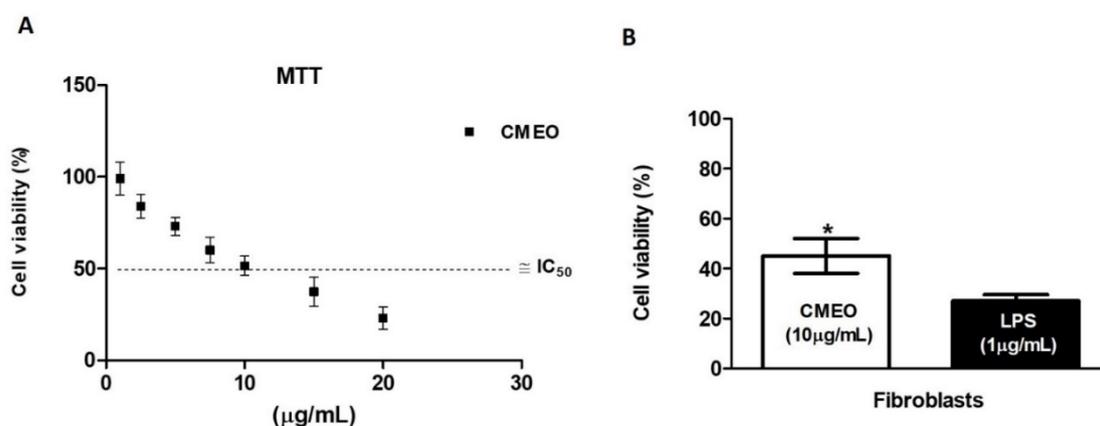
After the MTT assay and the determination of the $IC_{50\%}$, the subsequent aim was to investigate whether the CMEO was, in fact, able to increase collagen and hyaluronic acid synthesis. The **FIGURE 2** show the effects

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of CMEO on collagen (**FIGURE 2A**) and hyaluronic acid (**FIGURE 2B**) synthesis. In both cases, the synthesis from CCD1072Sk fibroblast cells were found to have significantly increased when compared to control (non-treated cells).

FIGURE 2: The effects of CMEO on collagen (A) and hyaluronic acid (B) synthesis.
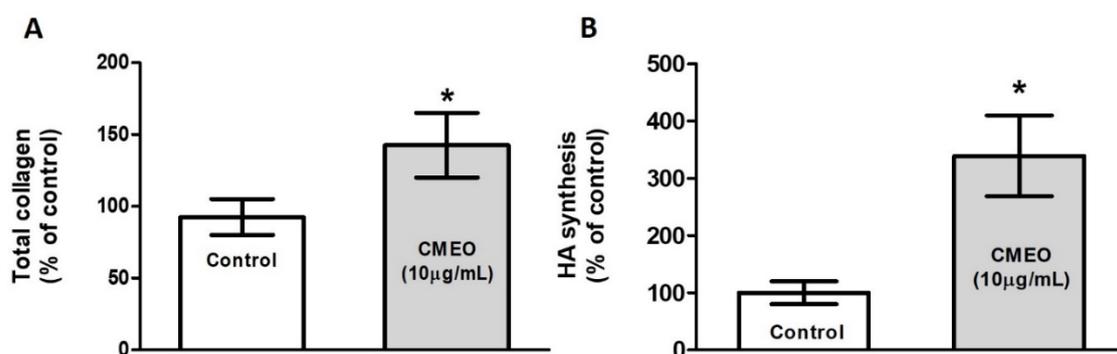
Legend: (A) The collagen synthesis was determined by Sirius red assay. Fibroblast cells were pre-treated in serum free medium for 24 hours and were further treated with CMEO (10 µg/mL) for 24 hours. The cells were stained with Sirius Red dye and the absorbance was determined using a microplate reader at 570 nm. (B) The hyaluronic synthesis was determined using an ELISA kit. Fibroblast cells were pre-treated in serum free medium for 24 hours and were further treated with CMEO (10 µg/mL) for 48 hours. The cultured medium was collected for ELISA and the absorbance was measured at 450 and 570 nm using a microplate reader. All assays have been performed in triplicate and the mean ± standard deviations are shown as $*p < 0.05$ versus the non-treated cells.

It is well known that collagenases are the enzymes that digest collagen. Therefore, the reduction of collagenase activity could protect against collagen breakdown. Thus, the effects of CMOE on collagenase activity showed that the essential oil dramatically decreased after treating the CCD1072Sk fibroblast cells (**FIGURE 3**).

FIGURE 3: The effects of CMOE on collagenase/MMP-1 activity was determined using a collagenase activity colorimetric assay kit.
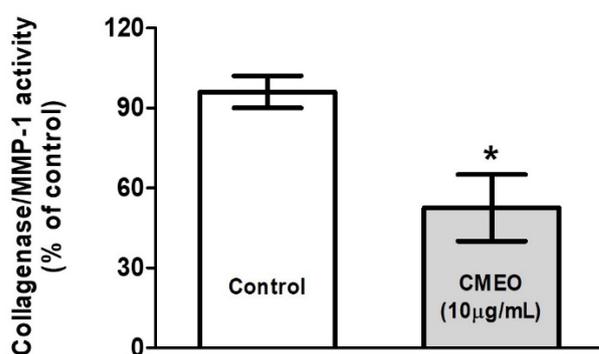

Legend: Fibroblast cells were pre-treated in serum free medium for 24 hours and were further treated with CMEO (10 µg/mL) for 48 hours. The cultured medium was collected, and the absorbance was measured at 345 nm using a microplate reader. All assays have been performed in triplicate and the mean ± standard deviations are shown as $*p < 0.05$ versus the non-treated cells.

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The **FIGURE 4 (A, B, AND C)** indicates that MMP-1 (collagenase), MMP-2, and MMP-9 mRNA enzymes were highly expressed in the LPS-activated fibroblasts cells. CMEO at 10 µg/mL significantly and similarly inhibited the mRNA expression of the metalloproteinases (MMP´s) enzymes as shown in the same **FIGURE 4 (A, B, AND C)**. At the same time, the effects of CMEO (10 µg/mL) and LPS on the levels of MMP-1, MMP-2, and MMP-9, were evaluated, as shown in **FIGURES 4 (D, E, AND F)** in conditioned medium from fibroblasts cultures.

The cytokines, IL-1β and IL-6, and the chemokines MIP-1α and MCP-1 were highly expressed in LPS-activated cells, however, the treatment with CMEO (10 µg/mL) significantly inhibited the expression of these inflammatory mediators in activated CCD1072Sk cells (**FIGURE 5**). The CMEO used in this study showed a high geraniol content (**TABLE 1**).

FIGURE 4: Effects of CMEO on the expression of mRNA and secretion of MMP-1; MMP-2; and MMP-9 in fibroblast cell line in presence or absence of LPS.
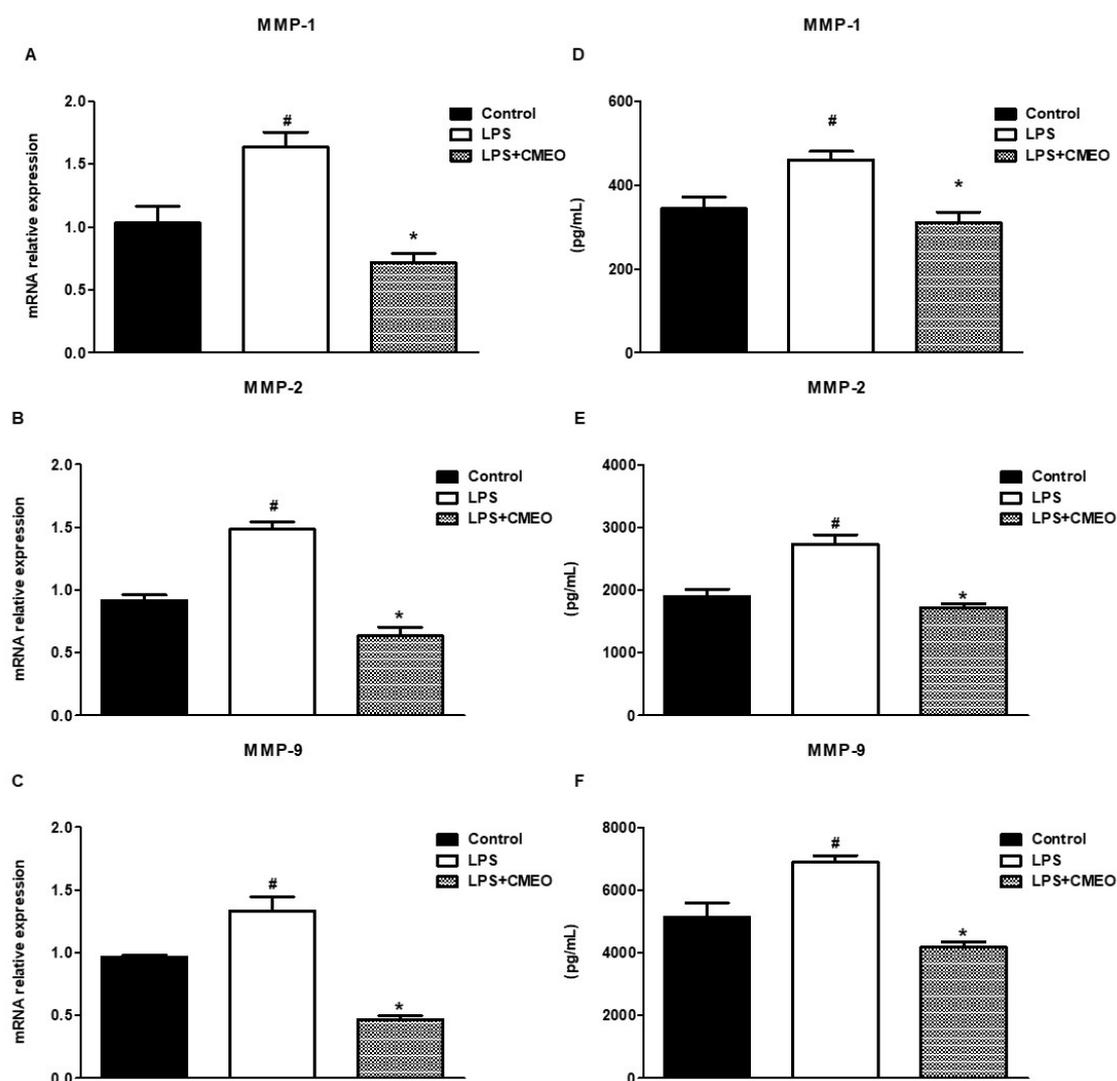
Legend: Cells were pretreated with CMEO (10 µg/mL) for 2 hours and treated with LPS (1 µg/mL) for 24 hours. (A) mRNA levels of MMP-1; (B) mRNA levels of MMP-2; and (C) mRNA of MMP-9. The mRNA levels were determined using real-time RT-PCR. ELISA analysis was performed to determine the levels of MMP-1 (A); MMP-2 (B); and MMP-9 (C) in the cell line culture supernatant. All assays have been performed in triplicate and the mean ± standard deviations are shown as *p < 0.05 versus LPS-treated cells.

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FIGURE 5: CMEO regulates LPS-induced release of inflammatory cytokine. Cells were pretreated with CMEO (10 µg/mL) for 2 hours and treated with LPS (1 µg/mL) for 24 hours. The effect of CMEO on decreasing release of interleukins and chemokines was determined by ELISA. All assays have been performed in triplicate and the mean ± standard deviations are shown as *p < 0.05 versus LPS-treated cells.
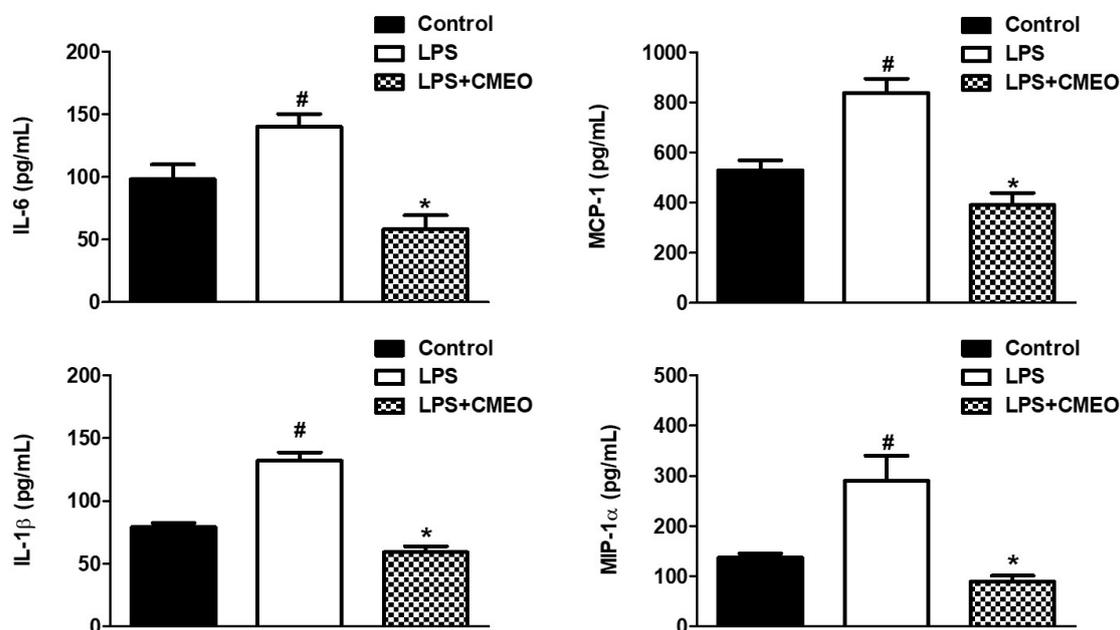
The major component found in CMEO was geraniol, corroborating data from the literature that indicate this as the main component of the essential oil of palmarosa (81,70%)[9-11]. In a screening carried out with different essential oils and V79 fibroblasts, it presented IC50% values between 19.50±5.96 and 448.00±19.52 µg/mL[11]. In a study with L929 fibroblasts tested with *Moringa oleifera* essential oil, it presented an IC50% of 42.99 ± 0.17 µg/mL[12].

An important biological factor associated with skin aging is the general atrophy of the extracellular matrix, reflected by a reduction in collagen levels[13]. In addition, collagen synthesis in skin fibroblasts plays a major role in skin rejuvenation[14], while the synthesis of hyaluronic acid, HA, an unsulfated glycosaminoglycan, regulates skin hydration, wrinkles and tissue repair[15]. Our results indicate that the CMEO significantly induced collagen and hyaluronic acid synthesis from the fibroblasts, suggesting that the CMEO could enhance skin moisture and can result in skin being less dry by increasing HA synthesis[16].

Our results indicate that CMEO, by reducing the secretion of IL-6, IL-1β, MCP-1, and MIP-1α, decreases the mRNA synthesis of MMPs that are produced by fibroblasts and stimulate the synthesis of total collagen. We suggest that CMEO, which present a high content of geraniol, could be part of cosmetic formulations, with antiaging indication, since, in addition to increasing collagen synthesis, it decreases the inflammatory response, which triggers the expression of MMPs, contributing to the degradation of matrix collagen extracellular, fundamental findings for skin aging. Finally, the results showed the potential of CMEO, probably due to the action of geraniol, as an important cosmetic ingredient, although further studies are needed to better understand the action and regulation of signaling pathways in different biological processes.

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MMP´s are enzymes that participate in the breakdown of elements in the extracellular matrix, playing an important role in homeostasis, aging and skin healing[17]. In addition to chronological aging, induced by UV radiation, increased production of MMPs, including MMP-1, MMP-2 and MMP-9, promotes changes in collagen synthesis by collagen induction or by degradation of the ECM[18]. The results obtained shows that the CMEO significantly reduced ($p<0.05$) the mRNA expression of the evaluated MMP´s, suggesting that CMEO could attenuate collagenolysis and elastolysis by matrix MMP´s that occur under normal conditions, and in most inflammatory processes[19]. The inflammatory response occurs, at least in part, by fragments of the proteolysis (e.g., elastin), which function as chemotactic agents, stimulating inflammation, proliferation, and angiogenesis[20].

The ability to reduce IL-6 secretion suggests that CMEO modulates collagenolytic effects through the negative modulation of this interleukin, where after induction by ultraviolet and infrared radiation, it stimulates, for example, the expression of MMP-1[21]. The reduction in the secretion of IL-1β, observed in treated fibroblasts with CMEO, can regulate the expression and activation of MMP´s[22], as MMP-2[23], and MMP-9[24]. Regarding the evaluated chemokines, the results obtained after the treatment of fibroblasts with CMEO, suggest that the reduction in the levels of MCP-1 and MIP-1α may reduce the expression of MMP´s, since results described in the literature, indicate an increase in MMP-1 and MMP-9 by these chemokines[25,26]. The degradation of collagen and glycosaminoglycans, promotes considerable deterioration of connective tissue leading to the appearance of wrinkles, reduced skin elasticity and firmness[27].

Geraniol is a natural acyclic monoterpene alcohol found in the essential oils of lemon grass, palmarosa, citronella, and other plants[28]. It is used in cosmetics, shampoos, and other non-cosmetic products[29]. Recently, geraniol was shown to possess various pharmacological properties, including antioxidant, antimicrobial, antitumor, and anti-inflammatory activities[30]. The effects presented, could be attributed, at least in part, to the action of geraniol, since its anti-inflammatory effect[31,32], indicates that this component of CMEO could it could negatively modulate the secretion of interleukins and chemokines, promoting less expression of enzymes related to collagenolysis and elastolysis, in addition to promoting an increase in total collagen synthesis. Thus, CMEO would act as a skin protector against the damages caused by chronological aging and/or induction by radiation exposure UV, since CMEO was not cytotoxic to fibroblast cell line, as well as in similar results described in the literature[33-35].

Conclusion

Our results showed that CMEO stimulated the synthesis of collagen and hyaluronic acid, reduced the secretion of inflammatory cytokines and negatively modulated MMP expression. These finds, associated with new studies, suggest a potential anti-aging, anti-scar, and would healing, especially in its inflammatory phase, from CMEO, through inflammatory control and possible prevention of degradation of the extracellular matrix. However, future studies with in vivo models should be conducted to validate the effects observed with CMEO.

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