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Avaliação da atividade anti-inflamatória do óleo essencial de *Citrus latifolia* Tanaka e limoneno em modelos experimentais em camundongos

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Sumário

Resumo

O gênero *Citrus* (Rutaceae) compreende diversas espécies de plantas produtoras de algumas das frutas mais cultivadas do mundo, dentre elas o limão, cujos frutos apresentam apreciável teor de óleo essencial. Na medicina popular os óleos essenciais do gênero *Citrus* são usados como tônicos, antitérmicos, colagogos, anti-inflamatórios, sedativos e antitóxicos e o óleo essencial do limão é utilizado como anti-séptico, diurético, carminativo e eupéptico. Plantas do gênero *Citrus* apresentam na constituição de seus óleos essenciais monoterpenos e sesquiterpenos, sendo o limoneno o maior constituinte. A atividade anti-inflamatória do óleo essencial do *Citrus latifolia* Tanaka (CLEO) e seu principal constituinte o limoneno (LIM) foram estudados, em diferentes modelos experimentais de inflamação aguda. No ensaio de viabilidade celular, o CLEO e o LIM apresentaram baixa citotoxicidade, em torno de 20%. Na peritonite induzida por zymosan, LIM inibiu significativamente o exsudato inflamatório e a infiltração de leucócitos. Na quimiotaxia *in vitro* tanto o CLEO, como o LIM, inibiram a migração de neutrófilos estimulada por diferentes agentes quimiotáxicos. Além disso, LIM reduziu o níveil da citocina TNF-α, e não alterou o nível de IL-10 no exsudato peritoneal. Nossos estudos indicam que CLEO e LIM apresentam atividade anti-inflamatória, provavelmente por inibir mediadores da inflamação envolvidos no edema inflamatório e na quimiotaxia de leucócitos. Novos estudos serão necessários para elucidar o mecanismo anti-inflamatório destas drogas.

Palavras-chave: *Citrus latifolia* Tanaka; quimiotaxia; óleo essencial; inflamação; limoneno.

Abstract:

The genus *Citrus* (Rutaceae) includes several species of plants producing some of most cultivated fruits in the world, which present an appreciable content of essential oil. In folk medicine they are used as digestive, tonic, antipyretic, cholagogue, anti-inflammatory, sedative and antitoxic. Lemon essential oil, since ancient times it is used as antiseptic, carminative, diurétic and eupeptic. In essential oils of plants of the genus *Citrus* have as constituents monoterpenes and sesquiterpenes, whereas limonene (LIM) is the main compound. This study, we investigated the anti-inflammatory activity of *Citrus latifolia* Tanaka essential oils (CLEO) and its main constituent LIM. In the cell viability assay the CLEO and LIM showed low cytotoxicity. In zymosan-induced peritonitis LIM present an reduced infiltration of leukocytes peritoneal exudate and decrease in the number of polymorphonuclear leukocytes, in *in vitro* chemotaxis CLEO and LIM promoted a significant reduction in the neutrophils migration toward fMLP and LTB_4. In addition, LIM reduced the TNF-α levels but not alter IL-10 levels in the peritoneal exudate. In conclusion our studies indicated that CLEO and LIM have anti-inflammatory activity, probably inhibiting inflammatory mediators present in the inflammatory exudate and leukocytes chemotaxis. Further studies are needed to elucidate the anti-inflammatory mechanism of these drugs.

Keywords: *Citrus latifolia* Tanaka; chemotaxis; essential oil; inflammation; limonene.

Evaluation of anti-inflammatory activity of *Citrus latifolia* Tanaka essencial oil and limonene in mice experimental models

1. Introduction

The genus *Citrus* (Rutaceae) includes several species of plants producing some of most cultivated fruits in the world, including orange and lemon, which present an appreciable content of essential oil. In folk medicine they are used as digestive, tonic, antipyretic, cholagogue, anti-inflammatory, sedative and antitoxic [1,2,3,4]. In essential oils of plants from genus *Citrus* have as constituents monoterpenes and sesquiterpenes [5,6]. Literature datas indicate the presence of 50 or more different compounds obtained from citrus peel, whereas limonene (LIM) is the main compound [3,7].

Lemon essential oil is a complex mixtures of chemical compounds such as: LIM, γ-terpinene, citral, linalool and β-caryophyllene, among other [8]. From ancient times it is used as antiseptic, carminative, diuretic and eupeptic [2]. Some of its compounds have anti-inflammatory activity reported, as β-caryophyllene, LIM and linalool, for example [9,10,11].

LIM is one of the most common terpenes in nature and has been used as flavoring agents in common food items, such as fruit juices, soft drinks, ice cream and also in the cosmetic and pesticides industries [10,12]. For LIM it was demonstrated antiulcerogenic, gastroprotective, chemopreventive, antiproliferative, insecticide, antimicrobial and immunomodulatory activities [13,14,15,16]. In addition, this compound was atributed anti-inflammatory activity by reducing eosinophil chemotaxis and MCP-1 production [10]. Also it was effective in inhibiting the lipopolysaccharide (LPS)-induced NO and prostaglandin E_2 (PGE_2) production in macrophage [17] and decreased interleukin-1 alpha (IL-1α) levels in normal human undifferentiated keratinocyte, NCTC 2544 [18].

The biological activity of extracts of herbs has been widely studied. However, there are few studies evaluating the effect of essential oils obtained from plants of the genus *Citrus* and its constituents on the anti-inflammatory activity. Then, in this study, we investigated the anti-inflammatory activity of *Citrus latifolia* Tanaka essential oils (CLEO) and its main constituent LIM.

2. Experimental

2.1. Plant material and essential oils

The fruits of *Citrus latifolia* Tanaka were purchased in the commerce Maringá-PR city. The essential oil was obtained from the flavedos of CLEO (690g), by hydrodistillation using a Clevenger-type apparatus and stored at temperature of 4°C in a refrigerator. The flavedos obtained using a grater stainless steel, were submitted to hydrodistillation by 2 hours. The oil was dried over sodium sulphate and stored in amber flask at 4°C. Yield of essential oil obtained was 2,76% v/w. The constituent limonene was isolated from CLEO.

2.2. Essential oil analysis

Gas Chromatography-Mass Spectrometry (GC-MS): GC analysis was performed with a Thermo Electron Corporation, Focus GC model, under the following conditions: DB-5 capillary column (30 m x 0.32 mm, 0.50 mm), column temperature, 60°C (1 min) to 180°C at 3°C/min; injector temperature 220°C; detector temperature 220°C; split ratio 1:10; carrier gas He; flow rate: 1.0 mL/min. Volume injected 1 µL diluted in Chloroform (1:10). The GC/MS analysis were performed in a Quadrupole mass espectrometer (Thermo Electron Corporation, DSQ II model), operating at 70 V. The identification of the individual components was based on comparison of their GC retention indices (RI) on apolar columns and comparison with mass spectra of authentic standard purchased from Sigma-Aldrich literature data (Adams, 2001).

Nuclear Magnetic Resonance (NMR): ^{13}C NMR (75.45 MHz) spectra was recorded in deuterated chloroform ($CDCl_3$) solution in a Mercury-300BB spectrometer, with δ (ppm) and spectra referred to $CDCl_3$ (δ 7.27 for ^{1}H and 77.00 for ^{13}C) as internal standard.

2.3 Animals

For the evaluation of the anti-inflammatory activity male Balb/C mice were used (20-25g). The animals were obtained from Central Animal House of the State University of Maringá and were housed at 22 ± 2ºC under a 12/12 h light/dark cycle. The experimental protocol was approved by the Ethical Committee in Animal Experimentation of the State University of

Maringá (CEAE/UEM066/2010).

2.4. Bioassays for cytotoxic activity

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay is based on the mitochondrial enzyme reduction of tetrazolium dye to detect and determine cell viability [19]. The neutrophils were obtained from the peritoneal cavity of Balb/C mice 4 hours after injection of 200 µL zymosan solution (1mg/cavity, i.p.). Briefly, the cells were plated at a density of $5X10^5$ cells/well in a volume of 100 µL RPMI medium (supplemented with 10% FCS and penicillin 100 U/mL + streptomycin 100 µg/mL) into 96-well plates. CLEO and LIM emulsion were prepared with RPMI and emulsified by sonication. After 90 min of cell exposure to CLEO and LIM: 3, 10, 30 and 90 µg/mL, 10 µL of MTT (5 mg/mL, Sigma) stock solution was added to each well. After 2 h incubation at 37 °C, the medium was removed and 100 µL of DMSO were added to each well. Cells were incubated at 25 °C for further 10 min and then the absorbance was read by a Biochrom Asys Expert Plus microplate reader (Asys®) at a wavelength of 540 nm. The values of the blank wells were subtracted from each well of treated and control cells, the percentage viability were determined as formulated below:

$$\% \text{ Viable cells} = \frac{\text{The absorbance of the treated cells} - \text{Absorbance of the blank}}{\text{The absorbance of the control - Absorbance of the blank}} \text{ X } 100 =$$

2.5. Anti-inflammatory activity

2.5.1. Zymosan-induced peritonitis in mice

In vivo neutrophil migration was performed in Balb/C mice. Mice were pretreated with LIM (125, 250 or 500 mg/kg, *p.o.*) or 0,2% aqueous Tween 80 solution (0.1 mL, *p.o.*) as the control. Thirty minutes later, all animals received an Zymosan injection (1mg/cavity) intraperitoneal stimulus or equivalent volume of vehicle (saline), 6 hours after the animals were killed, and the cells present into peritoneal cavity were harvested by introducing 2.0 mL of phosphate-buffered saline (PBS) containing EDTA. Then, counts were performed in total and differential cell. The results were expressed as the number of neutrophils.

2.5.2. In vitro chemotaxis assay

To evaluate CLEO and LIM effects on chemotaxis, neutrophils were isolated from the peritoneal cavity of Balb/C mice, 4 hours after Zymosan injection (1mg/cavity, i.p). The cell number was adjusted to $1x10^6$ cells/mL in RPMI/ BSA 0.1%. The chemotaxis assay was performed using a 48-well microchamber (Neuro Probe, Boyden chamber). The stimuli, fMLP (10^{-6}M) or LTB_4 (10^{-8}M), and negative control (RPMI 1640) were added to the lower chambers. A 5 μm pore polycarbonate membrane (Neuro Probe) was placed between the upper and lower chambers, and $1x10^6$ neutrophils pretreated with CLEO (1, 3 or 10 μg/mL) or LIM (1, 3 or 10 μg/mL) for 30 min were added to the top chambers. CLEO and LIM emulsion were prepared with RPMI and emulsified by sonication. Chambers were incubated at 1 h at 37 °C, 5% CO_2. Following incubation, the membrane was washed and stained using the *Instant Prov* (Newprove). Neutrophils were counted by optical microscopy (1000X), five fields in each well. The results were expressed as the number of neutrophils per field. Data were presented as mean ± SEM of 3 separate experiments.

2.5.3 Measurements of cytokine levels by ELISA

The levels of TNF-α and IL-10 were determined in peritoneal exudate, in Balb/C mice. The group of mice was pretreated with LIM (500 mg/kg, *p.o.*) or 0,2% aqueous Tween 80 solution (0.1 mL, *p.o.*) as the control. Thirty minutes later, all animals received an Zymosan injection (1mg/cavity) intraperitoneal stimulus or equivalent volume of vehicle (saline), 6 hours after the animals were killed, and exudate present in the peritoneal cavity were harvested by introducing 1.0 mL of phosphate-buffered saline (PBS) containing EDTA. The samples were centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was separated for dosing and rapidly frozen and stored at -70°C for later analysis. We used commercial kits for ELISA, according to the manufacturer's recommendations (R & D Systems ®, Cayman Chemical ®).

2.6 Statistical analysis

Data were expressed as the mean ± SEM for each group. Results were statistically analyzed by using One-way variance analysis (ANOVA) followed by Tukey's test and Student's *t* test. Differences were considered significant when $p< 0.05$.

3. Results and discussion

The genus *Citrus* (Rutaceae) consists of several plant species whose fruits have an appreciable content of essential oil. The C*itrus* essential oils have in its constitution monoterpenes and sesquiterpenes [1,5,6,7]. LIM is monoterpene found in a variety of plants, particularly in oils of lemon and orange. This monoterpene has antitumoral, antibiotic, antioxidant and antiprotozoal activity reported [12,13]. This study we investigated the anti-inflammatory effect of CLEO and its main constituent LIM.

The chemical composition of the CLEO was investigated by GC-MS and NMR. The results of the CG-MS analysis (Table A.1 and Fig. A.1) showed a predominance of limonene (62%), γ-terpinene (14.2%), β-pinene (12.2%), α-pinene (2.8%) and p-cymene (1.8%) similar to previous studies [3,6,7]. The percentage of each constituent and their retention indexes were summarized in Table A.1. In order to confirm the structure of the main compounds, the CLEO was studied by ^{13}C NMR (Fig. A.2). The chemical shift of each carbon in the experimental spectrum was compared with those of the spectra of pure compounds.

In the cell viability assays CLEO or LIM were tested in differents concentrations. CLEO in concentrations of 3, 10, 30 and 90 μg/mL showed a cell viability of 85%; 79%; 75% and 77%, respectively. LIM, in the same concentrations, showed a cell viability of 88%; 78%; 77% and 79%, respectively. Our data showed that CLEO and LIM treatments did not alter the cell viability, showed low cytotoxicity, with viability above 75% at concentration of 10 μg/mL, as observed in similar cytotoxicity study [3,12].

Many inflammatory mediators are involved in leukocytes migration such as: chemokines, leucotrienes, inflammatory cytokines and prostaglandins [20,21]. Essential oil treatment were effective in reduce chemotaxis, as observed for *Zingiber officinale* Roscoe, *Rosmarinus officinalis* L., *Cordia verbenacea* and *Perargonium asperum* essential oils [22,23,24,25].

Acute inflammatory reactions can be induced experimentally by a variety of substances. Zymosan, the insoluble polysaccharide component of the cell walls of Saccharomyces

cerevisiaeis is commonly used for induction of acute peritonitis in mice. In the zymosan-induced inflammatory processes several cytokines as tumor necrosis factor (TNF) and interleukin-6 (IL-6) are released and activation of the complement cascade brings about to neutrophil accumulation and vascular abnormalities [26,27].

To evaluate the effect of pretreatment with LIM on the migration of inflammatory cells *in vivo*, was held zymosan-induced peritonitis. After 6 h of peritonitis induction, an intense inflammatory response was characterized by an increase in the number of leukocytes peritoneal exudate (14,65 ±2,08 x 10^6 cells/cavity), compared with the control group (5,25±0,59 x 10^6 cells/cavity). The animals pretreated with LIM (500 mg/kg) present an reduced infiltration of leukocytes peritoneal exudate significantly compared to untreated animals (Fig. B.1). The decrease in the number of leukocytes is due mainly to a reduction in the number of polymorphonuclear leukocytes (Fig. B.2).

The formyl peptide, formyl-Met-Leu-Phe (fMLP), is a bacterial product that is recognized by neutrophils, upon binding to its heterotrimeric G protein-coupled receptor, that initiates signaling cascades that activate multiple pathways, these pathways include the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3K) cascades, which are important for the development of the functional responses of neutrophils in inflammation [28,29]. To avaluate the direct effect of CLEO and LIM on *in vitro* neutrophil chemotaxis, different concentrations were performed. The chemoattractant fMLP (10^{-6}M) or Leukotriene B_4 (LTB_4) - 10^{-8}M were used. CLEO at doses of 1, 3, and 10 µg/mL promoted a significant reduction ($p<0.05$) in the neutrophils migration toward fMLP stimuli: 31.32; 40.85; 45.45%, respectively (Fig. C.1) and the LIM treatment in the same doses, showed a significant reduction ($p<0.05$) toward fMLP stimuli: 38.68; 82.14; 87.63%, respectively (Fig. C.2).

LTB_4 is a potent chemoattractant derived from arachidonic acid, modulating diverse functions in living systems, and for instance, induces chemotaxis. The biological effects of LTB_4 are mediated pathways specific G-protein-coupled receptors, BLT1 and BLT2 activating the pathways of PI-3K [30,31]. CLEO at doses of 1, 3, and 10 µg/mL promoted a significant reduction ($p<0.05$) in the neutrophils migration toward LTB_4: 32.86; 34.80; 54.84%, respectively (Fig. C.2). In the same manner to that after LIM treatment: 29.48; 36.82; 34.52%, respectively (Fig. C.3). Since, LTB_4 and fMLP, were used as chemotaxis agents in *in vitro* tests, and CLEO and LIM also inhibited neutrophil migration, our results allow us to suggest that prostanoids and

cytokines are involved in this process.

Recent studies reinforce these results, showing that lemon essential oil inhibits the activity of 5-lipoxygenase (5-LOX) and the inhibitory effect of LIM could also be observed in eotaxin-induced chemotaxis by eosinophils [10,32]. Neutrophils have many cell surface receptors that are coupled to PI3K-dependent processes, including chemotaxis receptors [33]. In an under-agarose assay, neutrophils predominantly migrated towards the chemoattractants fMLP via p38 MAPK, whereas LTB_4-induced migration (intermediary chemoattractant) the migration was PI3K dependent [34,35].

The results presented in chemotaxis assay showed that both CLEO and LIM promoted significant inhibition of chemotaxis induced by stimulation with fMLP and LTB_4. However, pre-incubation of neutrophils with LIM promoted a more intense inhibition of migration induced by fMLP compared to that of CLEO. Since, in the leukocyte migration fMLP-induced involves prostanoids release [36], and the probably action mechanism of these substances could be related to the inhibition of cyclooxygenase - COX 1 and COX 2.

Various constituints of essential oils effectivelly inhibit cytokine production, as described for, 1,8-cineol that inhibited TNF-α and IL-1β in human lymphocytes; α-humulene reduced TNF-α production; terpinen-4-ol suppressed the production of TNF-α, IL-1β, IL-8, IL-10, and PGE_2 by LPS-activated monocytes for example [10,37,38,39]. The inflammatory response includes the recruitment of leukocytes and the release of inflammatory cytokine such as TNF- α, IL-1, IL-6, IL-10 and others [20, 40]. In this work, the levels of TNF-α and IL-10 in peritoneal exudate were determined and LIM (500 mg/kg, *p.o.*) and significantly inhibited TNF-α levels but not of IL-10 as shown in Fig. D.1 and D.2. Other studies show that the *Citrus* essential oil/magnesium salt mixture reduced TNF-α levels at the inflammatory site, and *Citrus* essential oil alone did not reduce the levels of IL-10 in the same study [41]. LIM has activity anti-inflammatory by reducing PGE_2 production in macrophage [17] and IL-1-α levels in normal human undifferentiated keratinocyte, NCTC 2544 [18]. Other compounds present in CLEO also have some anti-inflammatory activities, such as linalool that inhibits *in vitro* NO formation [11]; β-caryophyllene reduces expression of TNF-α, IL-1β, interferon-γ, and keratinocyte-derived chemokine [9]; and α-terpineol inhibits the gene expression of the IL-6 receptor [42].

4. Conclusion

In conclusion, our studies indicated that CLEO and LIM have anti-inflammatory activity probably inhibiting inflammatory mediators presents in the inflammatory exsudate and leukocytes chemotaxis. Further, studies are needed to elucidate the anti-inflammatory mechanism these drugs.

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Table A.1

Percentage chemical composition of the *Citrus latifolia* Tanaka essential oil

Retention time	Compound	Percentual (%)	identification
8.08	solvent*	--	MS[a]
8.90	α-pinene	2.8	MS, NMR[b]
10.14	β-pinene	12.2	MS, NMR
10.59	solvent*	-	MS
11.54	*p*-cymene	1.8	MS, NMR
11.72	limonene	62.0	MS, NMR
12.62	γ-terpinene	14.2	MS, NMR
13.50	linalool	0.9	MS, NMR
13.83	neral	1.6	MS, NMR
15.55	geranial	0.6	MS, NMR
17.80	----------	0.6	no identified
20.40	α-terpineol	1.4	MS, NMR
28.29	----------	0.6	no identified
24.8	β-caryophyllene	1.7	MS

*Chloroform ($CHCl_3$)

[a] Mass Spectrometry

[b] Nuclear Magnetic Resonance

1 **2**

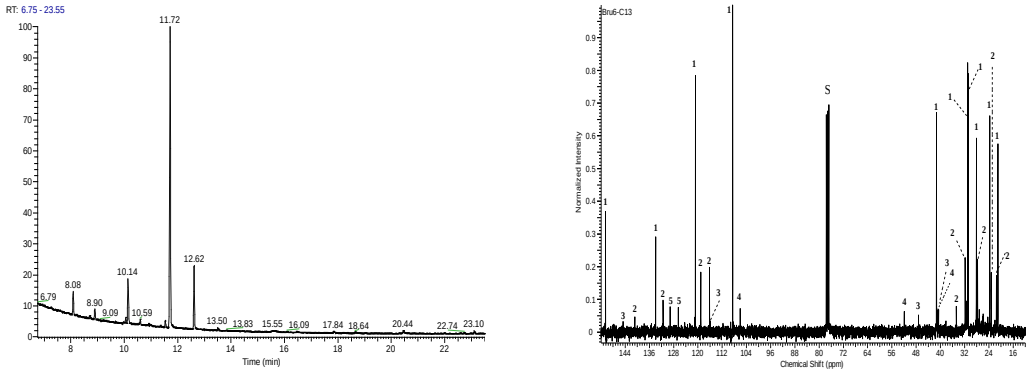

Fig. A. (1) GC chromatogram of the *Citrus latifolia* Tanaka essential oil. **(2)** ^{13}C NMR spectra of the *Citrus latifolia* Tanaka essential oil in deuterated chloroform ($CDCl_3$). The numbers on the peaks are attributed to majority compounds: 1. Limonene, 2. γ-Terpinene, 3. β-Pinene, 4. α-Pinene, 5. *p*-Cymene. S = solvent Chloroform (CHCl3).

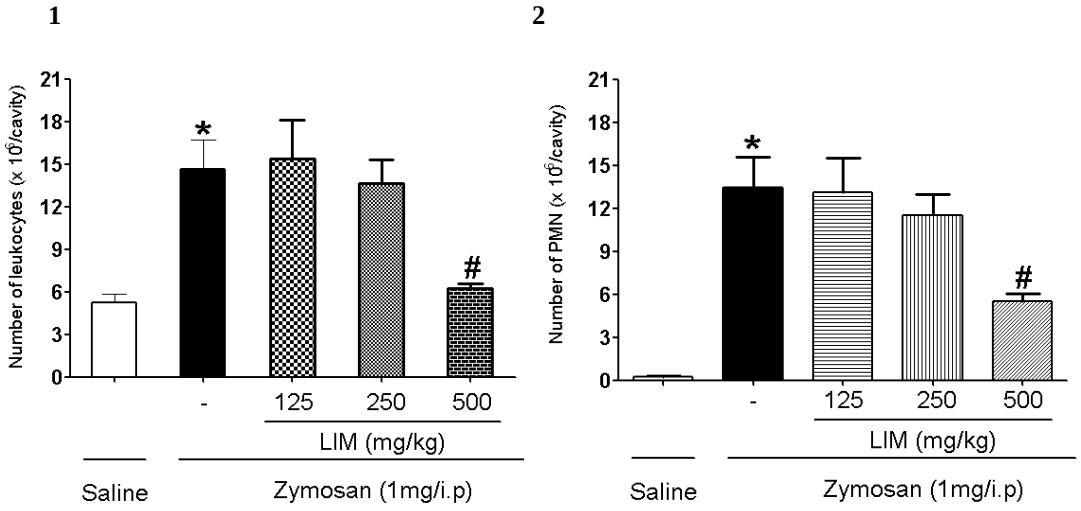

Fig. B. Effect of Limonene (LIM) treatments on leukocyte number, 6 hours after Zymosan injection (1mg/cavity/i.p) in Balb/C mice **(1);** and on PMN number **(2)**. *$p<0.05$ versus Saline (vehicle). $^{\#}p<0.05$ compared versus control group. (One-way ANOVA, Tukey's test).

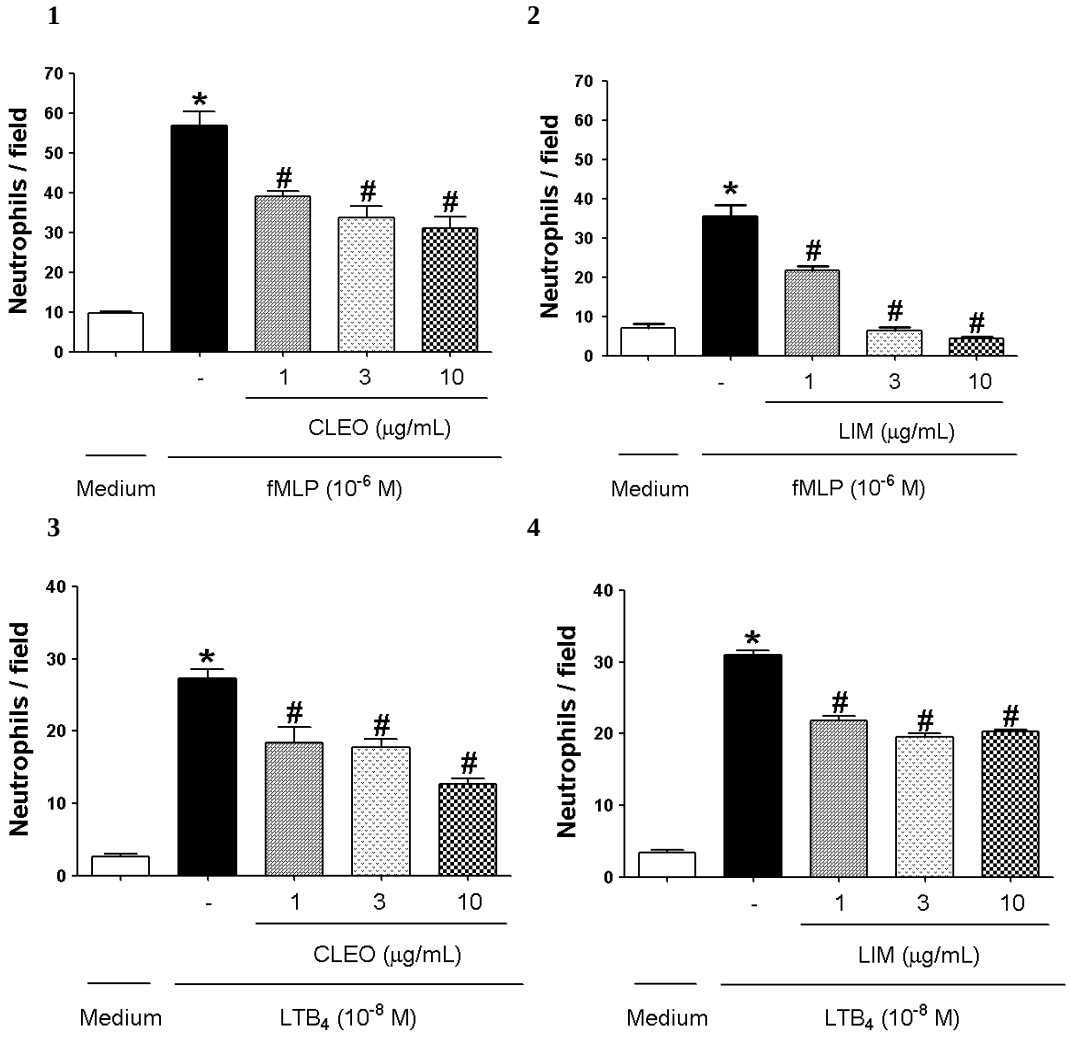

Fig. C. Effect of CLEO and LIM on neutrophils chemotaxis *in vitro*. Neutrophils were obtained from zymosan-induced peritonitis (1mg/cavity,) and stimulated with fMLP (10^{-6}) or LTB_4 (10^{-8}) after 30 min of treatment with CLEO (**1,3**) or LIM **(2,4)** at doses of 1, 3 and 10 µg/ml. Values are mean ± S.E.M. (n = 5) and are representative of three independent experiments. *$p<0.05$ versus Medium (RPMI 1640). $^{\#}p<0.05$ versus group of neutrophils stimulated with fMLP or LTB_4. (One-way ANOVA, Tukey's test).

1 **2**

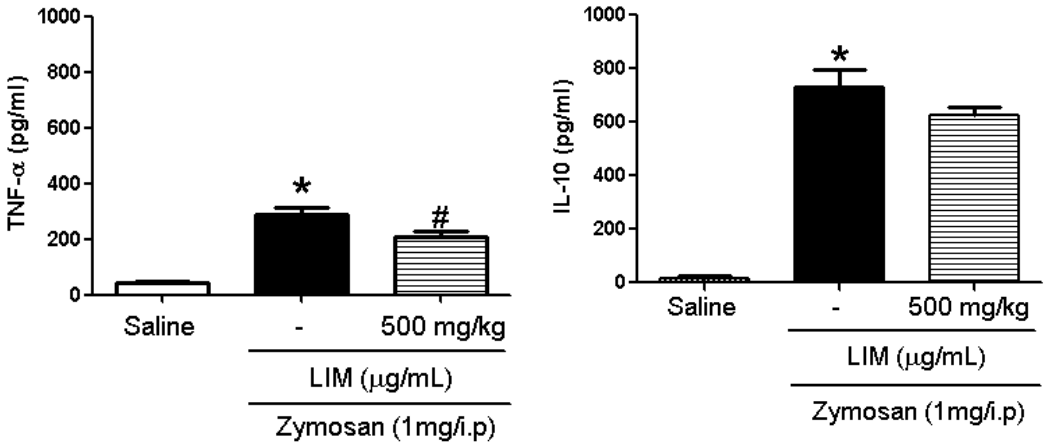

Fig. D. Effect of LIM on levels of TNF-α determined in peritoneal exudate, 6 hours after Zymosan injection (1mg/cavity) in Balb/C mice **(1)**; and on levels of IL-10 **(2)**. *$p<0.05$ versus Saline (vehicle). $^{\#}p<0.05$ compared versus control group. (Student's *t* test).