Journal of Ethnopharmacology 137 (2011) 909–913

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Lemongrass and citral effect on cytokines production by murine macrophages

Tatiana Fernanda Bachiega, José Maurício Sforcin*

Department of Microbiology and Immunology, Biosciences Institute, UNESP, Botucatu, SP, 18618-970, Brazil

ARTICLE INFO

Article history:
Received 18 March 2011
Received in revised form 9 June 2011
Accepted 6 July 2011
Available online 18 July 2011

Keywords:
Cymbopogon citratus
Lemongrass
Citral
Immunomodulation
Cytokines

ABSTRACT

Ethnopharmacological relevance: Cymbopogon citratus (DC) Stapf (Poaceae-Gramineae), an herb commonly known as lemongrass (LG), is an important source of ethnomedicines as well as citral, the major constituent of *Cymbopogon citratus*, used in perfumery, cosmetic and pharmaceutical industries for controlling pathogens. Thus, the goal of this work was to analyze the effect of LG and citral on cytokines production (IL-1β, IL-6 and IL-10) *in vitro*, as well as before or after LPS incubation.
Materials and methods: Peritoneal macrophages from BALB/c mice were treated with LG or citral in different concentrations for 24 h. The concentrations that inhibited cytokines production were tested before or after macrophages challenge with LPS, in order to evaluate a possible anti-inflammatory action. Supernatants of cell cultures were used for cytokines determination by ELISA.
Results: As to IL-1β, only citral inhibited its release, exerting an efficient action before LPS challenge. LG and citral inhibited IL-6 release. *Cymbopogon citratus* showed inhibitory effects only after LPS challenge, whereas citral prevented efficiently LPS effects before and after LPS addition. Citral inhibited IL-10 production and although LG did not inhibit its production, the concentration of 100 μg/well was tested in the LPS-challenge protocol, because it inhibited IL-6 production. LG inhibited LPS action after macrophages incubation with LPS, while citral counteracted LPS action when added before or after LPS incubation.
Conclusion: LG exerted an anti-inflammatory action and citral may be involved in its inhibitory effects on cytokines production. We suggest that a possible mechanism involved in such results could be the inhibition of the transcription factor NF-κB.

1. Introduction

Cymbopogon citratus (DC) Stapf (Poaceae-Gramineae), an herb commonly known as lemongrass (LG), is an important source of ethnomedicines. The tea from its leaves has been widely used as an antiseptic, antifever, antidyspeptic, carminative, tranquilizer and stomachic agent (Barbosa et al., 2008). Several studies have also demonstrated the anti-inflammatory, antiseptic, diuretic, neurobehavioral, antimicrobial, and fungistatic activities of *Cymbopogon citratus* (Carbajal et al., 1989; Francisco et al., 2011). Citral (3,7-dimethyl-2,6-octadienal) is the major constituent of *Cymbopogon citratus* and has been used in perfumery, cosmetic and pharmaceutical industries for controlling pathogens (Guynot et al., 2003).

Macrophages are one of the first lines of host defense. These cells undergo a series of physiological changes in response to infections or exposure to pathogen-derived products such as lipopolysaccharide (LPS). Upon activation, macrophages are more adept to kill pathogens. These cells are an important source of inflammatory cytokines, which represent an important strategy of host defense (Cao et al., 2006). As an example, interleukin (IL)-1β, a pro-inflammatory cytokine, is a key mediator of inflammation, inducing fever and the acute-phase response. IL-1β has important functions in the innate immune defense against microbes, trauma and stress, and is also an effector molecule involved in tissue destruction and fibrosis (Mandrup-Poulsen et al., 2010).

IL-6 is a potent and pleiotropic regulatory cytokine that mediates a plethora of physiological functions. IL-6 is known to influence cell growth, differentiation and migration during immune responses, hematopoiesis and inflammation (Frick et al., 2010).

Overproduction of proinflammatory cytokines may cause immunopathologies while defective production of these cytokines results in uncontrolled infection. Macrophages may control the overproduction of pro-inflammatory cytokines by producing anti-inflammatory cytokines such as IL-10, since it can inhibit the transcription and translation of a variety of inflammatory cytokines, reduce antigen presentation and inhibit or bias T cell activation (Anderson and Mosser, 2002).

Several studies have reported the traditional use of *Cymbopogon citratus*, but little is known about their influence on the immune system. In a previous work we reported that the treatment of mice with LG water extract inhibited macrophages to produce IL-1β and IL-6 production, suggesting the anti-inflammatory action of this spice *in vivo* (Sforcin et al., 2009). Thus, this study aimed to investigate the potential immunomodulatory effect of *Cymbo-*

* Corresponding author. Tel.: +55 14 3811 6058; fax: +55 14 3811 6058236.
E-mail address: sforcin@ibb.unesp.br (J.M. Sforcin).

0378-8741/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2011.07.021

pogon citratus on cytokines production (IL-6, IL-1β and IL-10) by peritoneal macrophages *in vitro*. After, the concentrations of *Cymbopogon citratus* that inhibited cytokines production were tested before or after macrophages challenge with LPS. Citral effects were also evaluated, in order to investigate a possible compound responsible for *Cymbopogon citratus* action.

2. Materials and methods

2.1. Lemongrass extract

The aerial parts of *Cymbopogon citratus* were collected in the Lageado Experimental Farm, UNESP, Campus of Botucatu. The plant material was identified and a voucher specimen (BOTU 25663) was stored in the Herbarium Botu of the Department of Botany, UNESP, Campus of Botucatu.

Fresh plant material was air-dried at 40 °C for 48 h. The dried leaves (400.0 g) were powdered in a blender and submitted to maceration for 72 h in 4 L of aqueous methanol 70% (v/v) at room temperature. The macerated material was filtered and concentrated in rotaevaporator. This dried extract was lyophilized and specific dilutions were prepared in RPMI media for each assay.

2.2. Citral

Citral was kindly provided by Dr. Jairo K. Bastos, School of Pharmaceutical Sciences of Ribeirão Preto, USP, Brazil. Specific dilutions of citral were prepared in RPMI media for the experiments.

2.3. Animals and peritoneal macrophages

Male BALB/c mice weighing 25–30 g and aged between 8 and 12 weeks were used. Mice were kept in rooms at 21–25 °C and 50% relative humidity, with a 12 h light/dark cycle. Food and water were provided *ad libitum*.

Peritoneal macrophages were obtained by inoculation of 3–5 mL of cold PBS in abdominal cavity. After a soft abdominal massage for 30 s, the peritoneal liquid was collected and put in sterile plastic tubes (Falcon). This procedure was repeated 3 or 4 times for each animal and the tubes were centrifuged at 200 × *g* for 10 min. Cells were stained with neutral red (0.02%), incubated for 10 min at 37 °C and counted in a haematocytometer to obtain a final concentration of 2×10^6 cells/mL. Cells were resuspended in cell culture medium (RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES – Sigma, USA) and cultured in a 96-well flat-bottomed plate (Corning, USA) at a final concentration of 2×10^5 cells per well. Cells were incubated at 37 °C and, after 2 h, non-adherent cells were removed (Sforcin, 2007).

This work agrees with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and was approved in August 8, 2008 (protocol n° 46/08-CEEA).

2.4. Cytotoxicity assay

Prior to *in vitro* assays, cells were incubated with LG extract or citral at the concentrations 5, 10, 25, 50 and 100 μg/well, in order to carry out the assays only with noncytotoxic concentrations. The evaluation of cytotoxicity was carried out by crystal violet method (Ait-Mbarek et al., 2007).

The macrophage culture received the stimuli as previously described, and after 24 h, supernatants were removed and 100 μL 0.5% crystal violet solution was added to the cells. After 10 min incubation at room temperature, the plates were washed and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulphate. Optical densities (OD) were read at 492 nm in an ELISA reader, and the percentage of cell viability was calculated using the formula: [OD test/OD control] × 100. Assays were carried out in triplicate.

2.5. In vitro assays

Macrophages were incubated with LG or citral at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h, supernatants were harvested and stored at −70 °C for cytokines (IL-1β, IL-6 and IL-10) measurement. Concentrations that inhibited IL-1β and IL-6 production followed two other protocols, challenging the cells with LPS.

2.5.1. Lemongrass and citral incubation before LPS challenge

Macrophages were pre-treated with LG (100 μg/well) or citral (25, 50 and 100 μg/well for IL-1β; 5, 10, 25, 50 and 100 μg/well for IL-6 and IL-10) at the concentrations that inhibited IL-1β, IL-6 and

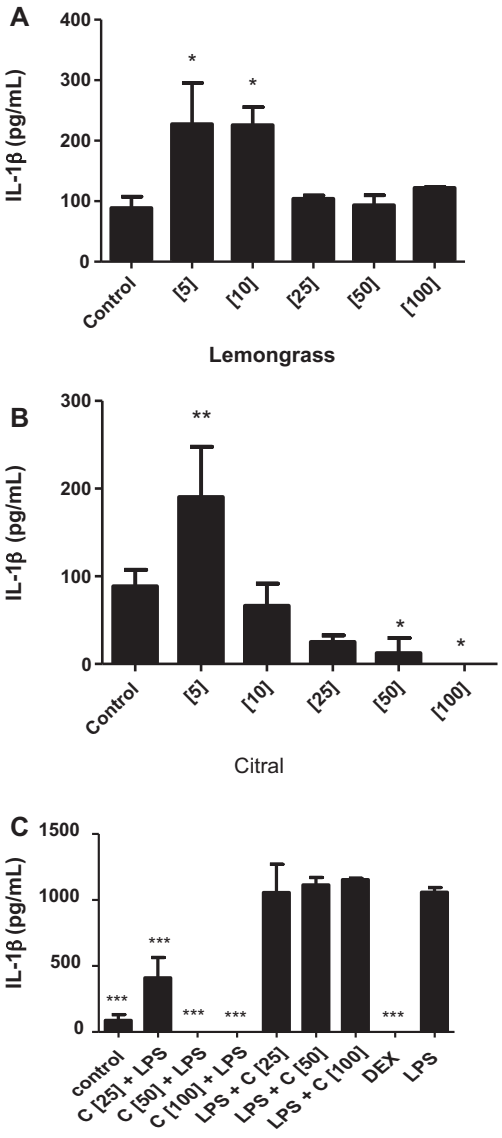

Fig. 1. IL-1β production (pg/mL) by peritoneal macrophages incubated with (A) lemongrass (LG) or (B) citral (C) at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37 °C. (C) IL-1β production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with C (25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/mL) were used as negative and positive control, respectively. *Significantly different from control ($P < 0.01$); **significantly different from control ($P < 0.001$); ***significantly different from LPS ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5–7 similar assays.

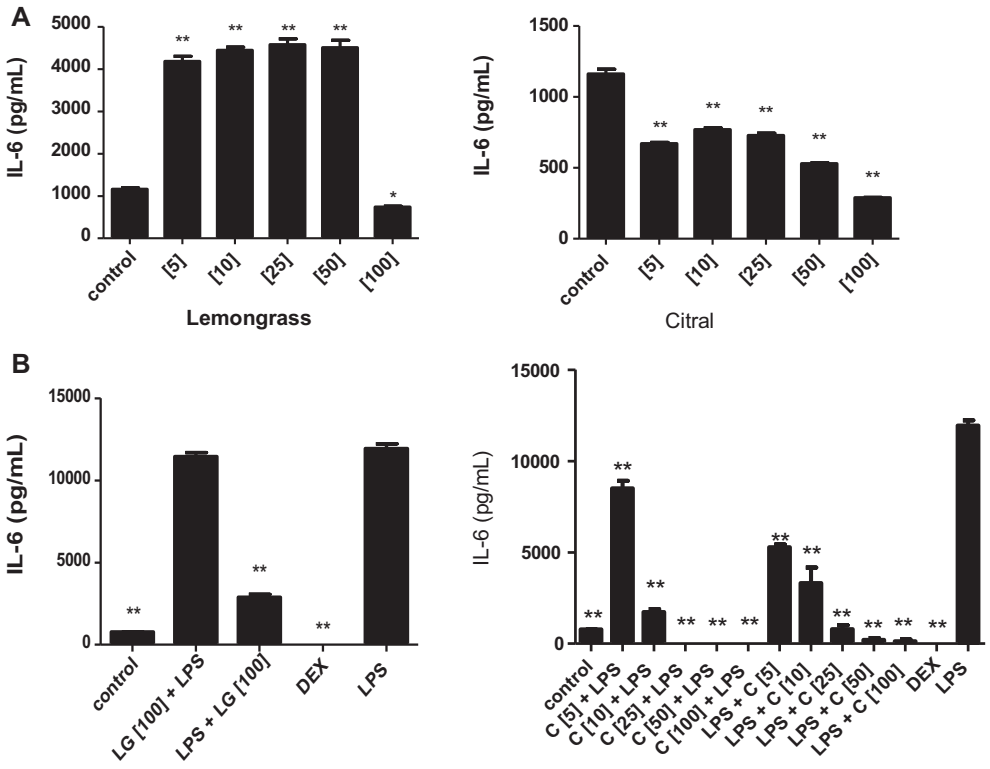

Fig. 2. (A) IL-6 production (pg/mL) by peritoneal macrophages incubated with lemongrass (LG) or citral (C) at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37 °C. (B) IL-6 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with LG (100 μg/well) and C (5, 10, 25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/mL) were used as negative and positive control, respectively. *Significantly different from control ($P < 0.001$); **significantly different from control or LPS ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5–7 similar assays.

IL-10 production for 2 h and then incubated with LPS (5 μg/mL) for 22 h. After this period, the culture supernatants were harvested and stored at −70 °C for cytokines measurement (Shin et al., 2006).

2.5.2. Lemongrass and citral incubation after LPS challenge

Macrophages were stimulated with LPS (5 μg/mL) for 2 h and then incubated with LG (100 μg/well) or citral (25, 50 and 100 μg/well for IL-1β; 5, 10, 25, 50 and 100 μg/well for IL-6 and IL-10) for 22 h. Afterwards, supernatants were collected and stored at −70 °C for cytokines determination (Shin et al., 2006).

Dexamethasone (DEX, 10^{-4} mol/L) (Zhuo et al., 2010) and LPS (5 μg/mL) were used as a negative and positive control, in order to inhibit and stimulate cytokine production, respectively.

2.6. Determination of cytokine production

IL-1β, IL-6 and IL-10 production was measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, USA). Briefly, a 96-well flat bottom Nunc Maxisorp (Nunc/Apogent, USA) was coated with a capture antibody specific to each cytokine. The plate was washed and blocked before 100 μL of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan et al., 2006).

2.7. Statistical analysis

Data were expressed as means ± standard-deviation of 5–7 similar assays. Analysis of variance (ANOVA) was used, followed by Dunnet's multiple comparison method, with 0.05 was chosen as the significant level (Zar, 1999).

3. Results

3.1. Cytotoxicity assay

Cymbopogon citratus extract and citral did not affect cell viability as determined by crystal-violet test (data not shown).

3.2. Cytokines production

3.2.1. IL-1β production

As to IL-1β, *Cymbopogon citratus* (5 and 10 μg/well) stimulated significantly its production ($P < 0.01$), whereas the concentrations 25, 50 and 100 μg/well did not affect its production ($P > 0.05$) (Fig. 1A). Citral (5 μg/well) stimulated this cytokine production ($P < 0.001$), whereas 10 and 25 μg/well did not affect its production ($P > 0.05$) and 50 and 100 μg/well inhibited significantly its release ($P < 0.01$) (Fig. 1B).

Thus, in the next protocol, since citral exerted an inhibitory action in IL-1β production (50 and 100 μg/well), macrophages were challenged with LPS before or after incubation with citral. The concentration of 25 μg/well was also included in this assay, since it diminished IL-1β production, although non-significantly. As seen in Fig. 1C, citral prevented LPS action, exerting an efficient action before LPS challenge ($P < 0.0001$). *Cymbopogon citratus* extract was not evaluated in LPS-challenge assays since it did not inhibit IL-1β production.

3.2.2. IL-6 production

IL-6 production was significantly ($P < 0.0001$) increased after *Cymbopogon citratus* incubation for 24 h (5, 10, 25 and 50 μg/well),

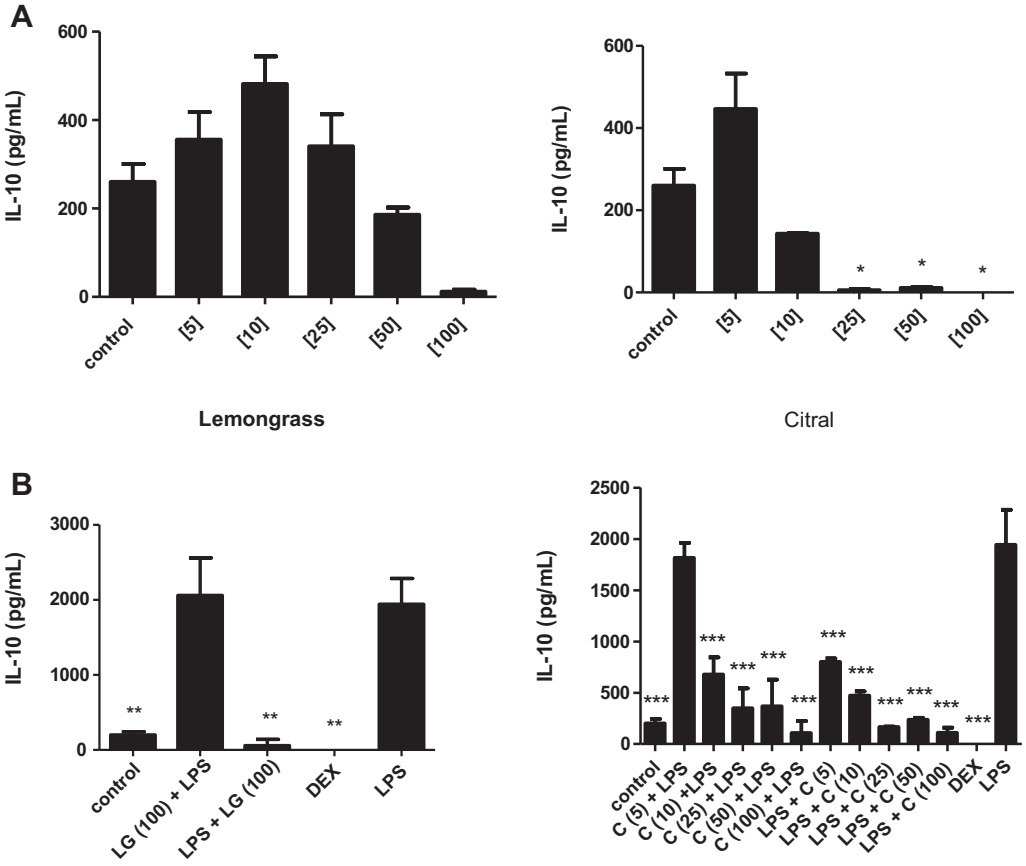

Fig. 3. (A) IL-10 production (pg/mL) by peritoneal macrophages incubated with lemongrass (LG) or citral (C) at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37 °C. (B) IL-10 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with LG (100 μg/well) and C (5, 10, 25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/mL) were used as negative and positive control, respectively. *Significantly different from control ($P < 0.01$); **significantly different from LPS ($P < 0.001$); ***significantly different from LPS (B) ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5–7 similar assays.

whereas 100 μg/well inhibited its production ($P < 0.001$) (Fig. 2A). Citral (5, 10, 25, 50 and 100 μg/well) decreased ($P < 0.0001$) IL-6 production by macrophages (Fig. 2A).

The inhibitory concentrations of *Cymbopogon citratus* (100 μg/well) and citral (5, 10, 25, 50 and 100 μg/well) were analyzed in the next protocol. Fig. 2B shows that *Cymbopogon citratus* showed inhibitory effects only after LPS challenge, and IL-6 production was lower than that induced by LPS alone ($P < 0.0001$). Citral prevented efficiently LPS effects before and after LPS addition ($P < 0.0001$) at all concentrations (Fig. 2B).

3.2.3. IL-10 production

There were no significant differences in IL-10 production by macrophages treated with LG ($P > 0.05$), whereas citral (25, 50 and 100 μg/well) inhibited its release ($P < 0.01$) (Fig. 3A).

Although lemongrass did not inhibit IL-10 production, the concentration of 100 μg/well was tested in the LPS-challenge protocol, because it inhibited IL-6 production. In fact, one may verify in Fig. 3B that LG (100 μg/well) inhibited significantly LPS action ($P < 0.001$), after macrophages incubation with LPS. Citral (5, 10, 25, 50 and 100 μg/well) counteracted significantly LPS action when added before or after LPS incubation ($P < 0.0001$) except when cells were incubated with citral (5 μg/well) and subsequently challenged with LPS ($P > 0.05$) when compared to LPS alone (Fig. 3B).

In all assays, DEX and LPS exerted their inhibitory and stimulatory activities, as negative and positive controls of cytokine production, respectively.

4. Discussion

Immunomodulators may be defined as agents that affect the immune system by regulating molecules, such as cytokines, hormones, neurotransmitters and others peptides, stimulating or inhibiting the events of the immune response (Spelman et al., 2006). In this study, we investigated the immunomodulatory action of *Cymbopogon citratus* and citral on cytokines production.

Previous works from our laboratory revealed that the treatment of mice with LG water extract inhibited macrophages to produce IL-1β and IL-6. LG essential oil inhibited these cytokines production by macrophages *in vitro* as well (Sforcin et al., 2009). These data encouraged us to investigate the effects of LG before and after macrophages challenge with LPS, in order to evaluate its efficacy as a possible anti-inflammatory agent, since there is a traditional use of LG for different purposes.

The chemical composition of *Cymbopogon citratus* (extract and essential oil) was previously described (Sforcin et al., 2009), and citral and linalool were the major constituents of our sample. Thus, we also evaluated citral effects in our assays, in order to investigate a possible involvement of this compound in *Cymbopogon citratus* action.

Lemongrass stimulated IL-1β production, whereas citral stimulated and inhibited its production depending on concentration. In our study, we included LPS-challenge protocols in order to observe a possible preventive or therapeutic action. Citral was more efficient to inhibit IL-1β production when incubated before LPS addition to the cells, suggesting its preventive activity.

LG stimulated and inhibited IL-6 production by macrophages, depending on concentration, while citral inhibited IL-6 release. In LPS protocols, LG was more efficient when added after LPS incubation, whereas citral seemed to be efficient either before or after LPS addition.

LG and citral inhibited IL-10 production. In LPS protocols, LG exerted a therapeutic action by counteracting LPS stimulatory action, while citral showed preventive and therapeutic effects.

Figueirinha et al. (2010) demonstrated that *Cymbopogon citratus* leaf infusion significantly inhibited other inflammatory parameters: nitric oxide (NO) production and inducible NO synthase expression by LPS-stimulated mouse skin dendritic cells, suggesting its anti-inflammatory activity. The pharmacological properties of citral were also investigated (Lertsatitthanakorn et al., 2006). Regarding its anti-inflammatory effects, Lee et al. (2007) reported that citral blocked LPS-induced activation of NF-κB by inhibiting the phosphorylation of IκB, which in turn inhibited p65 and p50 translocation to the nucleus – an initial process for gene expression of several cytokines. Anti-inflammatory effects of several natural products such as *Butea monosperma* and *Marasmius oreades* and their compounds gallatanins, butin, isobutin and butein have been reported to inhibit NF-κB activation (Lee et al., 2007; Rasheed et al., 2010), suggesting that the transcription factor NF-κB is an obvious target of anti-inflammatory therapeutics (Gadjeva et al., 2010).

Taken together, our data are in agreement with those found in literature, pointing out to the anti-inflammatory effects of citral, which may be involved in LG inhibitory effects on cytokines production, and a possible mechanism involved in LG and citral actions could be the inhibition of the transcription factor NF-κB. However, one should take into account that other chemical constituents in LG extract may affect cytokine production; moreover, herein we did not intend to compare the activities of citral and lemongrass, since citral concentration in the extract may be different from that of isolated citral used in our assays *in vitro*. Since inflammation plays an important role in host homeostasis, immunomodulatory agents may be useful to maintain or restore this balance. The potential of both LG and citral as immunomodulatory and anti-inflammatory agents should be further explored, in order to understand under which conditions they might be useful tools for prevention or treatment of inflammatory diseases.

Acknowledgements

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – 2008/06120-0) for the grant.

References

Ait-Mbarek, L., Ait-Mouse, H., Elabbadi, N., Bensalah, M., Gamouh, A., Aboufatima, R., Benharref, A., Chait, A., Kamal, M., Dalal, A., Zyad, A., 2007. Anti-tumor properties of blackseed (*Nigella sativa* L.) extracts. Brazilian Journal of Medical and Biological Research 40, 839–847.

Anderson, C.F., Mosser, D.M., 2002. Cutting edge: biasing immune responses by directing antigen to macrophage Fc_{γ} receptors. Journal of Immunology 168, 3697–3701.

Barbosa, L.C.A., Pereira, U.A., Martinazzo, A.P., Maltha, C.R.A., Teixeira, R.R., Melo, E.C., 2008. Evaluation of the chemical composition of Brazilian commercial *Cymbopogon citratus* (D.C.) Stapf samples. Molecules 13, 1864–1874.

Cao, S., Zhang, X., Edwards, J.P., Mosser, D.M., 2006. NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. Journal of Biological Chemistry 281, 26041–26050.

Carbajal, D., Casaco, A., Arruzazabala, L., Gonzalez, R., Tolon, Z., 1989. Pharmacological study of *Cymbopogon citratus* leaves. Journal of Ethnopharmacology 25, 103–107.

Figueirinha, A., Cruz, M.T., Francisco, V., Lopes, M.C., Batista, M.T., 2010. Anti-inflammatory activity of *Cymbopogon citratus* leaf infusion in lipopolysaccharide-stimulated dendritic cells: contribution of the polyphenols. Journal of Medicinal Food 13, 681–690.

Francisco, V., Figueirinha, A., Neves, B.M., García-Rodríguez, C., Lopes, M.C., Cruz, M.T., Batista, M.T., 2011. Cymbopogon citratus as source of new and safe anti-inflammatory drugs: bio-guided assay using lipopolysaccharide-stimulated macrophages. Journal of Ethnopharmacology 133, 818–827.

Frick, J.S., Grunebach, F., Autenrieth, I.B., 2010. Immunomodulation by semi-mature dendritic cells: a novel role of Toll-like receptors and interleukin-6. International Journal of Medical Microbiology 300, 19–24.

Gadjeva, M., Tomczak, M.F., Zhang, M., Wang, Y.Y., Dull, K., Rogers, A.B., Erdman, S.E., Fox, J.G., Carroll, M., Horwitz, B.H., 2010. A role for NF-{kappa}B subunits p50 and p65 in the inhibition of lipopolysaccharide-induced shock. Journal of Immunology 173, 5786–5793.

Guynot, M.E., Ramos, A.J., Setó, L., Purroy, P., Sanchis, V., Marín, S., 2003. Antifungal activity of volatile compounds generated by essential oils against fungi commonly causing deterioration of bakery products. Journal of Applied Microbiology 94, 893–899.

Lee, H.J., Jeong, H.S., Kim, D.J., Noh, Y.H., Yuk, D.Y., Hong, J.T., 2007. Inhibitory effect of citral on NO production by suppression of iNOS expression and NF-κB activation in RAW 264.7 cells. Archives for Pharmacal Research 31, 342–349.

Lertsatitthanakorn, P., Taweechaisupapong, S., Aromdee, C., Khunkitti, W., 2006. *In vitro* bioactivities of essential oils used for acne control. International Journal of Aromatherapy 16, 43–49.

Mandrup-Poulsen, T., Pickersgill, L., Donath, M.Y., 2010. Blockade of interleukin 1 in type 1 diabetes mellitus. Nature Reviews Endocrinology 6, 158–166.

Rasheed, Z., Akhtar, N., Khan, A., Khan, K.A., Haqqi, T.M., 2010. Butrin, isobutrin, and butein from medicinal plant Butea monosperma selectively inhibit nuclear factor-kappaB in activated human mast cells: suppression of tumor necrosis factor-alpha, interleukin (IL)-6, and IL-8. Journal of Pharmacology and Experimental Therapeutics 333, 354–363.

Sforcin, J.M., 2007. Propolis and the immune system: a review. Journal of Ethnopharmacology 113, 1–14.

Sforcin, J.M., Amaral, J.T., Fernandes Jr., A., Sousa, J.P.B., Bastos, J.K., 2009. LG effects on IL-1 and IL-6 production by macrophages. Natural Product Research 23, 1151–1159.

Shin, H.M., Lee, Y.R., Chang, Y.S., Lee, J.Y., Kim, B.H., Min, K.R., Kim, Y., 2006. Suppression of interleukin-6 production in macrophages by furonaphthoquinone NFD-37. International Immunopharmacology 6, 916–923.

Spelman, K., Burns, J., Nichols, D., Winters, N., Ottersberg, S., Tenborg, M., 2006. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. Alternative Medicine Review 11, 128–150.

Tan, E.L., Selvaratnam, G., Kananathan, R., Sam, C.K., 2006. Quantification of Epstein-Barr virus DNA load, interleukin-6, interleukin-10, transforming growth factor-β1 and stem cell factor in plasma of patients with nasopharyngeal carcinoma. BMC Cancer 6, 227.

Zar, J.H., 1999. Biostatistical Analysis, fourth ed. Prentice Hall, New Jersey.

Zhuo, Y.H., He, Y., Leung, K.W., Hou, F., Li, Y.Q., Chai, F., Ge, J., 2010. Dexamethasone disrupts intercellular junction formation and cytoskeleton organization in human trabecular meshwork cells. Molecular Vision 16, 61–71.