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Graphical abstract

In this report it was demonstrated that a lipid- and essential oil-free infusion of *Cymbopogon citratus* leaves (Cy), as well its polyphenols, have anti-inflammatory properties through inhibition of pro-inflammatory signaling pathways and nitric oxide production in lipopolysaccharide-stimulated macrophages. These evidences support the use of *Cymbopogon citratus* in traditional medicine and indicate that it could be a natural source of new and safe anti-inflammatory drugs.

Title
*Cymbopogon citratus* as source of new and safe anti-inflammatory drugs: bio-guided assay using lipopolysaccharide-stimulated macrophages

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Abstract

Ethnopharmacological relevance: Aqueous extracts of *Cymbopogon citratus* (Cy) leaves are used in traditional medicine for the treatment of inflammatory conditions, however little is known about their mechanism of action.

Aim of the study: The aim of this study is to explore the anti-inflammatory properties of *Cymbopogon citratus* leaves and their polyphenol-rich fractions (PFs), as well its mechanism of action in murine macrophages.

Materials and Methods: A lipid- and essential oil-free infusion of Cy leaves was prepared (Cy extract) and fractionated by column chromatography. Anti-inflammatory properties of Cy extract (1.115 mg/ml) and its PFs, namely phenolic acids (530 µg/ml), flavonoids (97.5 µg/ml) and tannins (78 µg/ml), were investigated using lipopolysaccharide (LPS)-stimulated Raw 264.7 macrophages as *in vitro* model. As inflammatory parameters, nitric oxide (NO) production was evaluated by Griess reaction, as well as effects on cyclooxygenase (COX-2), inducible NO synthase (iNOS) expression and on intracellular signaling pathways activation, which were analyzed by Western blot using specific antibodies.
Results: Cy extract inhibited iNOS expression, NO production and various LPS-induced pathways like p38 mitogen-activated protein kinase (MAPK), c-jun NH₂-terminal kinase (JNK) 1/2 and the transcription nuclear factor (NF)-κB. The extracellular signal-regulated kinase (ERK) 1/2 and the phosphatidylinositol-3-kinase (PI3K)/Akt activation were not affected by Cy extract. Both phenolic acid- and tannin-rich fractions significantly inhibited NF-κB activation, iNOS expression and NO production but none of the PFs modulated MAPKs or PI3K/Akt activation. Neither Cy extract or PFs affected LPS-induced COX-2 expression but LPS-induced PGE₂ production is inhibited by Cy extract and by phenolic acid-rich fraction.

Conclusions: Our data provide evidence that support the usage of Cymbopogon citratus leaves extract in traditional medicine, and suggest that Cy, in particular its polyphenolic compounds, could constitute a natural source of a new and safe anti-inflammatory drugs.

Keywords: Cymbopogon citratus, Poaceae-Gramineae, polyphenol, inflammation, nitric oxide, mitogen-activated protein kinases, nuclear factor-κB

Abbreviations: Cy, Cymbopogon citratus; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; FF, flavonoid-rich fraction; FSDC, fetal skin-derived dendritic cell; HPLC, high-performance liquid chromatography; Iκκ, IκB kinase; IκB, inhibitory protein κB; JNK, c-jun NH₂-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; PAF, phenolic acid-rich fraction; PFs, polyphenol-rich fractions; PGE₂, prostaglandin E₂; PI3K, phosphatidylinositol-3-kinase; SNAP, S-nitroso-N-acetylpenicillamine; TF, tannin-rich fraction; TLC, thin layer chromatography;
1. Introduction

Chronic inflammation is one of the leading causes of mortality in the western world and is associated with several pathologies like cancer (Porta et al., 2009), rheumatoid arthritis, diabetes (Schmidt and Duncan, 2003), cardiovascular and neurodegenerative diseases (Hunter and Doddi, 2010; Whitney et al., 2009). However, the current anti-inflammatory drugs have several limitations such as lack of responsiveness, side effects, delivery problems and cost of manufacture. Therefore, there is an urgent need to find new anti-inflammatory agents with selective pharmacology and less toxicity. Plant extracts have been used for centuries in traditional medicine to alleviate inflammatory diseases, however, and for some of them, little is known about their mechanisms of action. The understanding of molecular mechanisms behind the healing properties of natural products is crucial to find compounds that could be useful as templates to new therapeutic molecules. Indeed, most of the drugs actually available are derived from natural products (Newman and Cragg, 2007), therefore, the knowledge of phytochemicals molecular mechanisms became a good strategy in the search for new anti-inflammatory compounds.

In the inflammatory process, macrophages have a key role in providing an immediate defense against foreign agents. Upon activation with an inflammatory stimulus, such as lipopolysaccharide (LPS), macrophages produce a variety of pro-inflammatory mediators, including prostaglandin E$_2$ (PGE$_2$) and nitric oxide (NO) (Geller and Billiar, 1998). PGE$_2$ is synthesized by the rate limiting enzyme cyclooxygenase (COX), while NO is synthesized by nitric oxide synthase (NOS). Cyclooxygenase exists as two major isoforms (COX-1 and COX-2) and one variant (COX-3). While COX-1 is constitutively expressed in many tissues, COX-2 is an inducible enzyme expressed in the inflammatory-related cells, like macrophages, which produces large amounts of prostaglandins. In addition, LPS-activated macrophages also express transcriptionally inducible NOS (iNOS) that produces high amounts of NO from L-arginine. To date, three isoforms of NOS have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS. The high-output of NO by iNOS contributes to the pathogenesis of septic shock and inflammatory diseases (Guzik et al., 2003; Zamora et al., 2000). Therefore, the selective inhibition of COX-2 and iNOS in macrophages is a useful strategy to screen new anti-inflammatory drugs.

The expression of pro-inflammatory molecules is tightly regulated by several transcription factors and signaling pathways. Among these pathways, mitogen-activated proteins kinases (MAPKs) are signaling molecules that play critical roles in the regulation of cell growth, differentiation, cell survival/apoptosis and cellular response to cytokines and stress. The MAPKs pathways include p38 MAPK (Han et al., 1994), c-jun NH$_2$-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (Davis, 1994), and they are involved on LPS-induced COX-2 and iNOS expression in macrophages (Chen et al., 1999; Chen and Wang, 1999; Tsatsanis et al., 2006). Accordingly, it has been demonstrated that MAPKs inhibitors suppress the expression of iNOS gene (Chen et al., 1999). Besides, the iNOS expression could also be modulated by phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Salh et al., 1998), a serine/threonine kinase activated in response to certain growth factors and cytokines that provides a strong cell survival signal (Crawley et al., 1996; Gold et al., 1994). MAPKs and Akt also play
a critical role in the activation of nuclear factor (NF)-κB (Carter et al., 1999; Nakano et al., 1998). The NF-κB transcription factor regulates the expression of many genes involved in immune and inflammatory responses, including iNOS and COX-2 (Geller and Billiar, 1998). Many stimuli like LPS, cytokines and oxidants activate NF-κB through several signaling pathways that lead to the phosphorylation of inhibitory protein κB (IκB) by IκB kinase (Iκκ), which is a marker for ubiquitination and subsequent degradation by proteasome. IκB degradation unmasks the nuclear localization motif of NF-κB, which is rapidly translocated to the nucleus, where it activates the transcription of target genes. Therefore, the involvement of MAPKs, Akt and NF-κB in the regulation of inflammatory mediator’s synthesis makes them potential targets for novel anti-inflammatory therapeutics.

Cymbopogon citratus (DC) Stapf (Cy), Poaceae-Gramineae, commonly known as lemongrass, is a spontaneous perennial grass, largely distributed around the world, especially in tropical and subtropical countries. Its leaf essential oil citral is used in the food, perfumery, soap, cosmetic, pharmaceutical and insecticide industries (Negrelle and Gomes, 2007). Aqueous extracts of dried leaves are used in traditional medicine for the treatment of inflammation, digestive disorders, diabetes, nervous disorders, and fever, as well as other health problems (Carbajal et al., 1989; Lorenzetti et al., 1991). However, the mechanism of action of Cy is poorly explored and characterized, namely the mechanism responsible for its anti-inflammatory effects. We have previously demonstrated that Cy leaves extract has potent antioxidant activity that is related to its polyphenolic content (Figueirinha et al., 2008). In addition, we verified that this extract and its polyphenolic fractions inhibit LPS-induced NO production and iNOS expression in fetal skin-derived dendritic cell line (FSDC) (Figueirinha et al., 2010), reinforcing the potential use of Cy extract as source of a new anti-inflammatory drug.

Thus, this study aimed to explore the anti-inflammatory properties of Cymbopogon citratus extract by addressing its molecular mechanism of action. For that, we evaluated the effect of a lipid- and essential oil-free infusion (extract) obtained from Cy leaves and its polyphenol-rich fractions in COX-2 and iNOS expression, NO production and activation of MAPKs, Akt and NF-κB signaling pathways in vitro. As an in vitro model of inflammation, we used the mouse macrophage cell line, Raw 264.7, stimulated with LPS from E. coli.
2. Materials and Methods

2.1. Materials

LPS from *Escherichia coli* (serotype 026:B6) and the iNOS inhibitor, aminoguanidine, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Iscove’s Modified Dulbecco’s Medium, dexamethasone and wortmannin were from Sigma–Aldrich Química (Madrid, Spain). Fetal calf serum was purchased from Gibco (Paisley, UK). The protease and phosphatase inhibitor cocktails were obtained from Roche (Basel, Switzerland). SB203580, U0126, SP600125 and BAY 11-7082 were from Calbiochem (San Diego, CA, USA). Acrylamide was from Promega (Madison, WI, USA) and the polyvinylidene difluoride membranes were from Millipore Corporation (Bedford, MA). Antibodies against phospho-p44/p42 MAPK (ERK1/2), phospho-p38 MAPK, phospho-SAPK/JNK 1/2, phospho-Akt (Ser473) and IκB-α were from Cell Signaling Technologies (Danvers, MA, USA). The pan anti-JNK antibody was from R&D Systems (Minneapolis, MN, USA), the pan anti-p38 MAPK and Akt were from Cell Signaling Technologies (Danvers, MA, USA). The anti-actin and pan anti-ERK 1/2 antibodies were purchased from Millipore (Bedford, MA, USA). The alkaline phosphatase-linked secondary antibodies and the enhanced chemiluminescence reagent were obtained from GE Healthcare (Chalfont St. Giles, UK). All other reagents were from Sigma Chemical Co. (Saint Louis, MO) or from Merck (Darmstadt, Germany).

2.2. Plant material and extract preparation

Dry leaves of *Cymbopogon citratus* (Cy) Stapf. were purchased from ERVITAL® in July 2004 and kept at -20°C until used. The plant was cultivated in the region of Mezio, Castro D’Aire (Portugal). A voucher specimen was deposited in the herbarium of the University of Coimbra, Faculty of Pharmacy and J. Paiva (Botany Department, University of Coimbra, Portugal) confirmed the identity of the plant. An infusion was prepared from the powdered plant material (1:30 w/v), treated with *n*-hexane to remove lipids and essential oils and then freeze-dried (Cy extract). A yield of 16.6±1.2 g / 100 g of dry plant was obtained.

2.3. Extract fractionation

Cy extract was fractionated as previously described (Figueirinha et al., 2008) (Fig. 1). Briefly, the extract was treated with water and fractionated on a reverse phase semipreparative column Lichroprep® RP-18 (310 x 25 mm, particle sizes 40-63 μm), Merck (Darmstadt, Germany), eluted with water giving fraction F1 and with aqueous methanol solutions (fractions F2-F7). Dry residue of F7 was recovered in 50% aqueous ethanol and fractionated by gel chromatography on a Sephadex® LH-20 (Sigma-Aldrich – Amersham, Sweden) column (85 x 2.5 cm) using ethanol as mobile phase. All the
fractionation process described above was monitored by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) for polyphenols, providing three major fractions: tannin-rich fraction (TF; yield of 3.5% (w/w) of Cy extract) corresponding to F6, flavonoid-rich fraction (FF; yield of 4.4%(w/w) of Cy extract) corresponding to sub-fraction F7a, and phenolic acid-rich fraction (PAF; yield of 23.8% (w/w) of Cy extract) corresponding to F2 and sub-fraction F7b, as described in Figueirinha et al. (2010). The fractions were then taken to dryness under reduced pressure (40 °C). The Cy extract and the polyphenol-rich fractions were weighted in sterilized and humidity-controlled conditions, and then solubilized in endotoxin-free water.

**Fig. 1. Fractionation scheme of Cymbopogon citratus (Cy) extract.** Aqueous solution was fractionated on a reverse phase semi-preparative Lichroprep® RP-18 (310 x 25 mm, particle sizes 40-63 μm) and Sephadex® LH-20 (85 x 2.5 cm) columns, providing three major fractions: phenolic acid-rich fraction (PAF), flavonoid-rich fraction (FF), and tannin-rich fraction (TF).

### 2.4. Cell culture

Raw 264.7, a mouse leukaemic monocyte macrophage cell line from American Type Culture Collection, and kindly supplied by Dr. Otilia Vieira (Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Coimbra, Portugal), were cultured in Iscove’s Modified Dulbecco’s Eagle Medium supplemented with 10% non-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Along the experiments, cells were monitored by microscope observation in order to detect any morphological change.
2.5. Determination of cell viability by MTT assay

Assessment of metabolically active cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric assay as previously reported (Mosmann, 1983). Raw 264.7 cells (6x10^5 cells/well) were plated and allowed to stabilize for 12 h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with Cy extract, its polyphenolic fractions or with inhibitors for 1 h, and later activated with 1 µg/ml LPS for 24 h. After the treatments, a MTT solution (5 mg/ml in phosphate buffered saline) was added and cells incubated at 37 ºC for 15 min, in a humidified atmosphere of 95% air and 5% CO₂. Supernatants were then removed and dark blue crystals of formazan solubilized with acidic isopropanol (0.04 N HCl in isopropanol). Quantification of formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

2.6. Measurement of nitrite production by Griess reagent

The production of nitric oxide (NO) was measured by the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent (Green et al., 1982). Briefly, 170 µl of culture supernatants were diluted with equal volumes of the Griess reagent [0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H₃PO₄] and maintained during 30 min, in the dark. The absorbance at 550 nm was measured in an automated plate reader (SLT, Austria). Culture medium was used as blank and nitrite concentration was determined from a regression analysis using serial dilutions of sodium nitrite as standard.

2.7. Determination of nitric oxide scavenging activity using S-nitroso-N-acetylpenicillamine (SNAP) as NO donor

The nitric oxide scavenging activity was evaluated by incubating 1.115 mg/ml Cy extract, 530 µg/ml phenolic acid-rich fraction (PAF), 97.5 µg/ml flavonoid-rich fraction (FF), or 78 µg/ml tannin-rich fraction (TF) with 200 µM of NO donor SNAP, in culture medium during 3 h. After this period the nitrite levels in the medium were quantified by Griess method, as described above.

2.8. Measurement of prostaglandin E₂ (PGE₂) by enzyme immunoassay (EIA)

To analyse the production of PGE₂, Raw 264.7 cells (6x10^5 cells/well) were plated and allowed to stabilize for 12 h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with Cy extract or with its polyphenolic fractions, and later activated with 1 µg/ml LPS for 24 h. After the treatments, the supernatants were collected and frozen at -80ºC until the assay was
performed. The PGE$_2$ levels of diluted supernatants were quantified using an enzyme immunoassay (EIA) commercial kit from Cayman (Ann Arbor, MI, USA), following the manufacturer instructions.

2.9. Western blot analysis

To prepare total cell lysates for Western blot analysis, Raw 264.7 cells (24x10$^5$ cells/well) were plated and allowed to stabilize for 12 h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with Cy extract and its polyphenolic fractions for 1 h and then 1 µg/ml LPS was added for the indicated time. Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 2 mM ethylenediamine tetraacetic acid) freshly supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails and sonicated (four times for 4 s at 40 µm peak to peak) in Vibra Cell sonicator (Sonic & Material INC.) to decrease viscosity. The nuclei and the insoluble cell debris were removed by centrifugation at 4°C, at 12,000g for 10 min. The postnuclear extracts were collected and used as total cell lysates. Protein concentration was determined by the bicinchoninic acid protein assay and cell lysates were denaturated in sample buffer (0.125 mM Tris pH 6.8, 2% (w/v) sodium dodecyl sulfate, 100 mM dithiothreitol, 10% glycerol and bromophenol blue).

Western blot analysis was performed to evaluate the levels of iNOS and COX-2, and the activation of MAPKs, Akt and NF-κB signaling pathways. Briefly, equivalent amounts of protein were separated by 10% (v/v) SDS-PAGE followed by Western blotting. To examine the different proteins studied, the blots were incubated overnight at 4 ºC with the respective primary antibodies: COX-2 (1:10000), iNOS (1:7500), phospho-p38 MAPK (1:1000), phospho-JNK1/2 (1:1000), phospho-ERK 1/2 (1:1000), phospho-Akt (1:500) and total IκB (1:1000). Protein detection was performed using the enhanced chemifluorescence system and the membranes were scanned for blue excited fluorescence on the Storm 860 (GE Healthcare). The generated signals were analyzed using the software ImageQuant TL®. To demonstrate equivalent protein loading, membranes were stripped and reprobed with antibodies against the total form of MAPKs and Akt or with anti-actin antibody.

2.10. Statistical analysis

Results are expressed as mean±SEM of the indicated number of experiments. Statistical analysis comparing a treatment condition to control was performed between two groups and analyzed using two-sided unpaired $t$-test. When comparing the effect of different treatments to LPS-stimulated cells, a multiple group comparison was performed and one-way ANOVA followed by Dunnett's test was used. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). The significance level was *p <0.05, **p <0.01 and ***p <0.001, when compared to control and *p <0.05, **p <0.01 and ***p <0.001, when compared to LPS.
3. Results

3.1. Evaluation of the anti-inflammatory properties and molecular targets of lipid- and essential oil-free *Cymbopogon citratus* leaves infusion (Cy extract)

Some studies have been conducted with citral, the main volatile compound of the essential oil of *Cymbopogon citratus* (Cy) (Cheel et al., 2005; Lee et al., 2008), however little is known about the properties and mechanisms of action of the fixed compounds, namely polyphenols. Therefore, in the present study we analyzed their anti-inflammatory potential and evaluated some molecular targets of these extracts in LPS-stimulated Raw 264.7 cells. The Cy extract concentration used for this study was selected based on our previous results, obtained in dendritic cells (Figueirinha et al., 2010), and also on the absence of macrophages toxicity (table 1).

Table 1. Effect of Cy extract, polyphenol-rich fractions and signaling pathways inhibitors on macrophage cell viability. Raw 264.7 cells were treated with the indicated compounds for 24 h, and the cell viability was assessed as described in materials and methods. The results are expressed as percentage of control (non-treated cells) and each value represents the mean±SEM from at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell viability (% of control) mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
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<tr>
<td>Cy extract (1.115 mg/ml)</td>
<td>122.40±3.71</td>
</tr>
<tr>
<td>PAF (530 µg/ml)</td>
<td>82.24±3.95</td>
</tr>
<tr>
<td>FF (97.5 µg/ml)</td>
<td>84.69±4.09</td>
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<tr>
<td>TF (78 µg/ml)</td>
<td>89.33±5.80</td>
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<tr>
<td>LPS (1 µg/ml)</td>
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<td>LPS + Cy (1.115 mg/ml)</td>
<td>112.00±5.59</td>
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<tr>
<td>LPS + PAF (530 µg/ml)</td>
<td>97.00±7.15</td>
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<td>LPS + FF (97.5 µg/ml)</td>
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<td>LPS + SP600125 (20 µM)</td>
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<tr>
<td>Treatment</td>
<td>COX-2 Protein Levels (%)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>LPS + U0126 (10 µM)</td>
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<tr>
<td>LPS + Wortmannin (500 nM)</td>
<td>86.06±11.37</td>
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<tr>
<td>LPS + BAY 11-7083 (250 nM)</td>
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<tr>
<td>LPS + Aminoguanidine (50 µM)</td>
<td>95.18±8.21</td>
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</table>

3.1.1. Cy extract does not affect LPS-induced COX-2 expression but inhibits the PGE$_2$ production

We analyzed the effect of Cy extract on LPS-induced COX-2 expression after 24 h of murine macrophages stimulation by Western blot using a specific anti-COX-2 antibody (Fig. 2A). In non-stimulated Raw 264.7 cells (control), COX-2 protein was almost undetectable, but after LPS treatment the expression strongly increased to 10573±1544% of control ($p<0.001$). The LPS-induced COX-2 expression was not significantly inhibited by Cy extract (7824±1489% of control) while the extract alone was able to induce the expression of COX-2 (3226±579% of control).

Instead Cy did not inhibit the LPS-induced COX-2 expression, the enzyme activity could be compromised. Therefore, we next investigated the effect of Cy extract on a product of COX-2 activity, PGE$_2$, by enzyme immunoassay (EIA). As shown in Fig. 2B, the cell treatment with LPS induced a great increase in PGE$_2$ production, consistent with the results obtained for COX-2 expression, which is inhibited by macrophage pre-treatment with Cy (42.40% of inhibition). The Cy alone increased the LPS-induced PGE$_2$ production comparing to untreated Raw 264.7 cells (from 0.71±0.16 of control to 7.56±0.29 of Cy). Taken together, these results indicated that Cy extract did not inhibit the LPS-induced COX-2 activity, but modulates its activity, exhibiting anti-inflammatory properties, while the extract alone increased the COX-2 expression and the PGE$_2$ production.
Fig 2. Lack of effect of *Cymbopogon citratus* (Cy) extract on LPS-induced COX-2 expression and inhibition of LPS-induced PGE₂ production in murine macrophages. (A) Raw 264.7 cells (24x10⁵ cells) were maintained in culture medium (control), or pre-incubated with 1.115 mg/ml Cy extract for 1 h and then treated with 1 µg/ml LPS for 24 h. COX-2 expression was analyzed by Western blot using a specific anti-COX-2 antibody. An anti-actin antibody was used to confirm equal protein loading. The blot shown is representative of 3 blots yielding similar results. Results were expressed as percentage of COX-2 protein levels relatively to control. (B) Raw 264.7 cells (6x10⁵ cells) were maintained in culture medium (control), or pre-incubated with 1.115 mg/ml Cy extract for 1 h and then treated with 1 µg/ml LPS for 24 h. PGE₂ levels were evaluated in the culture supernatants by enzyme immunoassay (EIA), as described in material and methods, and the results expressed as percentage of LPS. Each value represents the mean±SEM from 2-3 independent experiments (**p<0.01, compared to control; ###p<0.001, compared to LPS).

3.1.2. Cy extract inhibits LPS-induced iNOS expression and nitrite production

We also investigated the effect of Cy extract on the production of the pro-inflammatory mediator NO, found in inflammatory disorders (Guzik et al., 2003). First, the effect of Cy in iNOS expression triggered by LPS was verified by Western blot (Fig. 3A). In untreated cells (control), iNOS protein expression is not detected but after treatment with LPS for 24 h, iNOS expression is strongly increased (1841±121.4% of control), as described earlier (Thiemermann, 1997). Pre-treatment of cells with Cy extract reduced the LPS-induced expression by 28.95% while extract alone slightly increased the iNOS expression (491.7±53.67% of control).
Secondly, the effect on NO production was analyzed by measuring accumulation of nitrite in the culture medium. As shown in Fig. 3B, untreated Raw 264.7 cells produced low levels of nitrites (2.115±0.7590 μM), consistent with the data obtained for iNOS expression in resting conditions. After cell activation with LPS for 24 h, the nitrite production increased to 46.67±2.623 μM, while macrophage pre-treatment with Cy strongly decreased the LPS-induced nitrite production (64.07% of inhibition). The Cy alone slightly increased nitrite production (10.59±1.691 μM). To evaluate Cy anti-inflammatory potency, a comparison with the known anti-inflammatory compound dexamethasone was performed. A decrease on LPS-induced NO production by Cy extract was verified in a magnitude similar to that observed for 20 μM dexamethasone (64.07% and 79.56%, respectively). We also analyzed the NO scavenging capacity of Cy extract, using SNAP as NO donor, and we found that Cy extract was no NO scavenging properties (data not shown). Taken together, these results suggest that Cy extract exhibit anti-inflammatory properties by inhibiting LPS-induced NO production while the extract slightly promoted NO production.
Fig 3. Inhibitory effect of Cymbopogon citratus (Cy) extract on LPS-induced iNOS protein expression and nitrite production in murine macrophages. (A) Raw 264.7 cells (24x10⁵ cells) were maintained in culture medium (control), or pre-incubated with 1.115 mg/ml Cy extract for 1 h and then treated with 1 µg/ml LPS for 24 h. iNOS expression was analyzed by Western blot using a specific anti-iNOS antibody and an anti-actin antibody was used to confirm equal protein loading. The blot shown is representative of 3 blots yielding similar results. Results were expressed as percentage of iNOS protein levels relatively to control. (B) Raw 264.7 cells (6x10⁵ cells) were maintained in culture medium (control), or pre-incubated with 1.115 mg/ml Cy extract or 20 µM dexamethasone for 1 h and then treated with 1 µg/ml LPS for 24 h. Nitrite levels in the culture supernatants were evaluated by the Griess reaction as described in material and methods. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. Each value represents the mean±SEM from at least 3 experiments (**p<0.01, compared to control; *p <0.05, ***p<0.001, compared to LPS).

At last, the signaling pathways involved in the modulation of NO production were investigated using specific inhibitors. The concentrations of these inhibitors were chosen based on the absence of cytotoxicity to macrophages (table 1). As shown in Fig. 4, the LPS-induced nitrite production was inhibited by SB203580 (53.59% of inhibition), a specific inhibitor of p38 MAPK, by SP600125 (65.40% of inhibition), a selective and reversible JNK inhibitor, by BAY 11-7082 (67.80% of inhibition), a NF-κB inhibitor, and by aminoguanidine (79.42% of inhibition), an inhibitor of iNOS. Both ERK 1/2 inhibitor (U0126) and PI3K/Akt inhibitor (wortmannin) were without effect on nitrite production.
3.1.3. Cy extract inhibits LPS-induced activation of p38 MAPK, JNK 1/2 and NF-κB

Our results demonstrated that LPS-induced NO production in macrophages was inhibited by Cy extract and regulated by p38 MAPK, JNK 1/2 and NF-κB signaling pathways but not by ERK 1/2 or PI3K/Akt. Therefore, we next evaluated the effect of Cy extract on the activation of those pathways by Western blot using phospho-specific antibodies. As shown in Fig. 5, LPS stimulation for 30 min induced the phosphorylation of Akt and all MAPKs, namely p38 MAPK, JNK 1/2 and ERK 1/2, as described previously (Rao, 2001). Pre-treatment with 1.115 mg/ml Cy extract inhibited the LPS-induced
phosphorylation of p38 MAPK and JNK 1/2 but had no effect in the activation of ERK 1/2 and Akt pathways. When added to control cells, Cy alone stimulated both MAPKs and Akt signaling pathways.

Since NF-κB transcription factor is a crucial player in the inflammatory process by controlling the expression of several pro-inflammatory genes, such as iNOS, an investigation of how NF-κB activation is affected by the Cy extract in LPS-activated macrophages was carried out measuring IκBα proteolytic degradation by Western blot. After 15 min of macrophages stimulation with LPS, we observed that IκBα was almost completely degraded (Fig. 5E). Pre-treatment of 1 h with 1.115 mg/ml Cy extract partially prevented the IκBα degradation induced by LPS and therefore the NF-κB activation. Taken together these data suggest that Cy extract selectively inhibits different LPS-induced pro-inflammatory signaling cascades.
Fig 5. Inhibitory effect of *Cymbopogon citratus* (Cy) extract on the LPS-activation of p38 MAPK, JNK 1/2 and NF-κB signaling pathways. Raw 264.7 cells (24x10^5 cells) were maintained in culture medium (control), or pre-incubated with 1.115 mg/ml Cy extract for 1 h and then treated with 1 μg/ml
LPS for 30 min to see the effect on MAPKs and Akt phosphorylation or for 15 min to see the effect on IκBα degradation. Total cell extracts were analyzed by Western blot using antibodies against (A) phospho-p38 MAPK, p38 MAPK, (B) phospho-JNK 1/2, JNK 1/2, (C) phospho-ERK 1/2, ERK 1/2, (D) phospho-Akt, Akt, (E) IκBα and actin. Each blot shown is representative of 3 blots yielding similar results.

3.2. Contribution of polyphenolic fractions, namely phenolic acid-, flavonoid- and tannin-rich fractions of Cymbopogon citratus leaves infusion to the Cy extract activity

Cy polyphenolic fractions inhibited the LPS-induced NO production and iNOS expression in dendritic cells (Figueirinha et al., 2010). So, we next evaluated the contribution of each polyphenolic fraction, namely phenolic acids (PAF) flavonoids (FF) and tannins (TF), to the effect of Cy extract in LPS-stimulated macrophages. The concentrations of the fractions used in this work were selected based on the absence of citotoxicity (table 1) and their ratios in the Cy extract after the fractionation: PAF (23.8%), FF (4.4%) and TF (3.5%).

3.2.1. Cy polyphenolic fractions do not affect COX-2 expression, but PAF inhibits PGE₂ production

First, we tested the effect of polyphenol-rich fractions in the LPS-induced COX-2 expression in Raw 264.7 macrophages. Similarly to the Cy extract, none of the fractions tested, PAF (530 μg/ml), FF (97.5 μg/ml) and TF (78 μg/ml), affected the macrophage COX-2 expression elicited by LPS (Fig. 6A).

Since Cy extract inhibited the PGE₂ production in LPS-stimulated macrophages, we next investigated the contribution of polyphenolic fractions to this activity. As shown in figure 6B, the LPS-induced PGE₂ production is strongly inhibited by PAF (35.17±2.47% of LPS), but not significantly affected by FF or TF (106.20±3.50% and 79.54±0.36% of LPS, respectively). These results indicated that PAF is partially responsible for the anti-inflammatory properties of Cy extract by inhibition of PGE₂ production.

The effect of Cy fractions on COX-2 expression and PGE₂ production in non-stimulated cells was also tested and none of the treatments interfered neither with the COX-2 expression either with PGE₂ production.
Fig 6. Lack of effect of polyphenol-rich fractions from Cy on LPS-induced COX-2 expression and inhibition of LPS-induced PGE\(_2\) production by PAF in macrophages. (A) Raw 264.7 cells (24x10\(^5\) cells) were maintained in culture medium (control), or pre-incubated for 1 h with 530 μg/ml phenolic
acid-rich fraction (PAF), or 97.5 μg/ml flavonoid-rich fraction (FF), or 78 μg/ml tannin-rich fraction (TF) and then treated with 1 μg/ml LPS for 24 h. Total cell extracts were analyzed by Western blot using a specific anti-COX-2 antibody and an anti-actin antibody was used to confirm equal protein loading. The blot shown is representative of 3 blots yielding similar results. Results were expressed as percentage of COX-2 protein levels relatively to control. (B) Raw 264.7 cells (6x10^5 cells) were treated as above. PGE_2 levels were evaluated in the culture supernatants by enzyme immunoassay (EIA), as described in material and methods, and the results expressed as percentage of LPS. Each value represents the mean±SEM from 2-3 independent experiments (**p<0.001, compared to control; *** p<0.001, compared to LPS).

3.2.2. Polyphenol-rich fractions inhibit LPS-induced iNOS expression and NO production

Since Cy extract inhibited iNOS expression and NO production in LPS-stimulated Raw 264.7 macrophages, the contribution of polyphenol-rich fractions to this activity was investigated. All fractions drastically decreased the expression of iNOS (Fig. 7A) and this inhibition was higher than that observed for the whole extract. PAF inhibited the LPS-induced iNOS expression by 75.37%, FF by 75.73% and TF by 86.34%, while Cy extract inhibited the iNOS expression by 28.95% (Fig. 3A). In addition, PAF and TF fractions significantly inhibited the LPS-induced nitrite production by 50.63% and 41.59%, respectively (Fig. 7B). Similarly to Cy extract, none of the fractions exhibit NO scavenging properties (data not shown). From these results, we can conclude that these fractions highly contribute to the anti-inflammatory properties of Cy extract. To note that, the fractions alone did not increase iNOS expression nor NO production, suggesting that polyphenolic compounds are not responsible for the slight pro-inflammatory properties observed with the Cy extract.
Fig 7. Inhibitory effect of polyphenol-rich fractions from Cy on LPS-induced iNOS expression and nitrite production. (A) Raw 264.7 cells (24x10^5 cells) were maintained in culture medium (control), or pre-incubated for 1 h with 530 μg/ml phenolic acid-rich fraction (PAF), or 97.5 μg/ml flavonoid-rich fraction (FF), or 78 μg/ml tannin-rich fraction (TF) and then treated with 1 μg/ml LPS for 24 h. Total cell
extracts were analyzed by Western blot using an anti-iNOS antibody and an anti-actin antibody was used to confirm equal protein loading. The blot shown is representative of 3 blots yielding similar results. Results were expressed as percentage of iNOS protein levels relatively to control. (B) Raw 264.7 cells (6x10^5 cells) were treated as above. Nitrite levels in the culture supernatants were evaluated by the Griess reaction as described in material and methods. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (μM) of nitrite in culture medium. Each value represents the mean±SEM from at least 3 experiments (**p<0.01, compared to control; ***p<0.001, compared to LPS).

3.2.3. Polyphenol-rich fractions inhibit LPS-mediated NF-κB activation but not MAPKs or PI3K/Akt signaling pathways

As Cy extract inhibited the LPS-induced p38 MAPK and JNK 1/2 activation, the contribution of polyphenol-rich fractions to the signaling pathways modulated by Cy was analyzed. As shown in Fig. 8, the polyphenol-rich fractions did not interfere significantly with the LPS-induced activation of MAPKs and Akt pathways, but inhibited the LPS-induced IkBα degradation. Overall, these results indicate that the polyphenolic fractions of *Cymbopogon citratus* are not responsible for the modulation of p38 MAPK and JNK 1/2; however they seem to be involved in the inhibition of LPS-induced NF-κB activation.
Fig 8. Inhibitory effect of polyphenol-rich fractions from Cy on LPS-activation of NF-κB signaling pathway. Raw 264.7 cells (24x10⁵ cells) were maintained in culture medium (control), or pre-incubated for 1 h with 530 μg/ml phenolic acid-rich fraction (PAF), or 97.5 μg/ml flavonoid-rich fraction (FF), or 78 μg/ml tannin-rich fraction (TF) and then treated with 1 μg/ml LPS for 30 min to see the effect on MAPKs and Akt phosphorylation or for 15 min to see the effect on IκBα degradation. Total cell extracts were analyzed by Western blot using antibodies against (A) phospho-p38 MAPK, p38 MAPK, (B) phospho-JNK 1/2, JNK 1/2, (C) phospho-ERK 1/2, ERK 1/2, (D) phospho-Akt, Akt, (E) IκBα and actin. Each blot shown is representative of 3 blots yielding similar results.
4. Discussion

In the course of screening anti-inflammatory compounds derived from plants, we previously demonstrated that *Cymbopogon citratus* (Cy) has strong antioxidant properties due to the presence of polyphenols (Figueirinha et al., 2008) and that Cy extract inhibits NO production and iNOS expression in dendritic cells (Figueirinha et al., 2010), suggesting an anti-inflammatory activity for this plant. The present study demonstrates that Cy extract, used in traditional medicine to treat inflammation and other health problems (Carbajal et al., 1989; Lorenzetti et al., 1991), has anti-inflammatory properties due to the selective inhibition of NO production through the pro-inflammatory signaling cascades p38 MAPK, JNK 1/2 and NF-κB, in murine macrophages.

Using LPS-stimulated macrophages as *in vitro* model, we demonstrated that Cy extract inhibited iNOS expression and NO production. Using pharmacological signaling pathways inhibitors, it was observed that the LPS-induced NO production is mainly controlled by p38 MAPK, JNK 1/2 and NF-κB pathways. Accordingly, previous studies demonstrated that JNK 1/2 (Zhōu et al., 2008) and p38 MAPK, but not ERK 1/2 (Chen and Wang, 1999), modulated iNOS expression and NO production in LPS-stimulated Raw 264.7 macrophages. In addition, activated MAPKs and PI3K/Akt were also implicated in NF-κB activation (Carter et al., 1999; Nakano et al., 1998), being NF-κB one of the critical transcription factors that controls iNOS gene expression in macrophages (Geller and Billiar, 1998). Cy extract also inhibited p38 MAPK, JNK 1/2 and NF-κB signaling pathways. Therefore, and since the signaling pathways involved in NO production are the same that Cy extract inhibited, the inhibition of NF-κB, p38 MAPK and JNK 1/2 pathways by Cy extract is probably responsible for its inhibitory effect on NO production. It was also observed that the iNOS inhibitor aminoguanidine almost abolished the nitrite production induced by LPS, indicating that in Raw 264.7 macrophages stimulated with LPS, the iNOS protein is the main, if not the only, NO producer. The effect of Cy extract on NO production was quite similar to that of the iNOS inhibitor aminoguanidine, almost abolishing the nitrite production induced by LPS, indicating that in Raw 264.7 macrophages stimulated with LPS, the iNOS protein is the main, if not the only, NO producer. The effect of Cy extract on NO production was quite similar to that of the iNOS inhibitor aminoguanidine, emphasizing its potent anti-inflammatory capacity and indicating that Cy extract inhibited NO production in part by inhibiting the iNOS expression. However, taking into account the higher effect in NO production relatively to the effect on iNOS expression, the Cy extract may also affect the levels of NO by other mechanisms. It was previously demonstrated that Cy extract had strong antioxidant properties (Cheel et al., 2005; Orrego et al., 2009), however we observed that Cy extract did not possess NO scavenging activity. Therefore, probably it affected the NO levels by other mechanisms than its antioxidant properties.

Cy extract has a high content in polyphenolic compounds (Figueirinha et al., 2008) that are secondary metabolites of plants with many healthy effects, including anti-inflammatory properties (Gonzalez-Gallego et al., 2007). Besides its antioxidant properties, recent data suggest that polyphenols could have other anti-inflammatory action mechanisms, namely, inhibition of iNOS, COX-2, MAPKs and NF-κB pathways and that the inhibitory mechanisms of polyphenols are not only signal specific, but also cell type dependent (Santangelo et al., 2007). Analyzing the effect of the polyphenol-rich fractions on iNOS expression and NO production we conclude that PAF and TF are the fractions responsible for the
inhibitory effect on NO production observed with the Cy extract. Probably, these fractions have a synergistic effect since the Cy extract has a little more activity than each fraction. Moreover, the polyphenolic fractions have a stronger inhibitory effect on iNOS expression. All the fractions inhibited iNOS expression while only PAF and TF inhibited NO production, suggesting that polyphenolic fractions modulate not only the iNOS expression but also its activity. Accordingly, recent studies demonstrate that polyphenols could modify the iNOS activity by modulating the availability of L-arginine, the rate-limiting substrate of iNOS (Mori and Gotoh, 2000). We also previously demonstrated that Cy extract and its polyphenols have iNOS and NO inhibitory properties in dendritic cells (Figueirinha et al., 2010). However, the polyphenol-rich fractions have different inhibitory capacity in dendritic and macrophage cells, indicating that the action of Cy polyphenols might be cell specific.

Many evidences reported that MAPKs signaling cascades might be differentially involved in the macrophage response to anti-inflammatory compounds (Choi et al., 2008; Lee et al., 2010; Park et al., 2008; Zhou et al., 2008). In order to explore the mechanisms underlying the inhibitory effect of polyphenol-rich fractions on NO production, phosphorylation levels of p38 MAPK, JNK 1/2, ERK 1/2 and Akt were analyzed by Western blot in LPS-stimulated Raw 264.7 macrophages. In contrast to Cy extract, none of the polyphenolic fractions inhibited MAPKs or PI3K/Akt pathways indicating that polyphenols are not involved in the inhibition of these pathways, being the compounds responsible for these effects eliminated during the fractionating procedure. Furthermore, we also observed that polyphenolic fractions inhibited the LPS-induced IκB degradation, suggesting that the inhibitory effect of the fractions on LPS-induced iNOS expression is due to inhibition of NF-κB activation, as described for other compounds (Cheng et al., 2001; Pan et al., 2000). Since NF-κB has an important role in inflammation and its inhibition is one of the main strategies to alleviate chronic inflammation, we can conclude that Cy extract, in particular their polyphenol-rich fractions, are a promising source of new anti-inflammatory drugs. In agreement, we are actually conducting more detailed work to better understand the modulation of NF-κB by Cy extract and to identify the compounds responsible for this effect, using bioguided assays.

In the present study it was showed that Cy extract inhibited PGE₂ production, being phenolic acid-rich fraction (PAF) responsible by this activity. However, neither Cy extract nor its polyphenolic fractions seemed to inhibit the LPS-induced COX-2 expression. Current treatment of inflammation is mainly based in non-steroid anti-inflammatory drugs (NSAIDs) that act by inhibiting COX-2. However, recent investigation points out that COX-2 specific inhibitors are associated with adverse renal and cardiovascular effects (Harirforoosh and Jamali, 2009; Ritter et al., 2009). Since FF and TF did not inhibit COX-2 expression neither its activity, they could be used as anti-inflammatory agents avoiding the secondary effects associated with COX-2 inhibition.

Intriguingly, Cy extract alone has a stimulatory effect, slightly increasing iNOS expression and NO production. This effect was due to the intrinsic properties of the extract and not due to the presence of endotoxins, since we obtained the same increase on NO production after application of the Cy extract in an endotoxin removal column (data not shown). However, in the LPS-stimulated macrophages the anti-
inflammatory properties of Cy overlay its pro-inflammatory activity, occurring inhibition of iNOS expression and NO production, as well as inhibition of p38 MAPK, JNK 1/2 and NF-κB activation, all these events being straightly connected with inflammation. In addition, the polyphenol-rich fractions did not show stimulatory effects and did not interfere with signaling pathways, indicating that the compounds responsible for the pro-inflammatory properties of the Cy extract are not the polyphenols contained in those fractions.

In conclusion, this paper demonstrates that a lipid- and essential oil-free infusion of *Cymbopogon citratus* leaves strongly inhibited the iNOS expression, NO production, p38 MAPK, JNK 1/2 and NF-κB signaling pathways in murine macrophages (Fig. 9), being the phenolic acids (PAF) and tannins (TF) responsible for its anti-inflammatory properties through inhibition of transcription factor NF-κB, iNOS expression and NO production. Taken together, these results provide evidence to understand the therapeutical effects of Cy extract, and suggest that its polyphenols might be a potential natural source of new anti-inflammatory drugs for the treatment of inflammatory disorders. However, further work is required to identify which compound(s) are responsible for the anti-inflammatory properties of *Cymbopogon citratus*, as well as the cellular and molecular mechanisms underlying these properties.

![Diagram](image)

**Fig 9.** Schematic model for the anti-inflammatory mechanism of lipid- and essential oil-free infusion of *Cymbopogon citratus* leaves (Cy extract) and its polyphenol-rich fractions on LPS-stimulated murine macrophages.
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Conflict of interest

None of the authors has any conflict of interest.
References


