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PII: S0378-8741(13)00247-X
DOI: http://dx.doi.org/10.1016/j.jep.2013.03.077
Reference: JEP8042

To appear in: *Journal of Ethnopharmacology*

Received date: 28 November 2012
Revised date: 22 March 2013
Accepted date: 28 March 2013

Cite this article as: Vera Francisco, Gustavo Costa, Artur Figueirinha, Carla Marques, Paulo Pereira, Bruno Miguel Neves, Maria Celeste Lopes, Carmen García-Rodríguez, Maria Teresa Cruz, Maria Teresa Batista, Anti-inflammatory activity of *Cymbopogon citratus* leaves infusion via proteasome and nuclear factor-κB pathway inhibition: contribution of chlorogenic acid, *Journal of Ethnopharmacology,* http://dx.doi.org/10.1016/j.jep.2013.03.077

Anti-inflammatory activity of *Cymbopogon citratus* leaves infusion via proteasome and nuclear factor-κB pathway inhibition: contribution of chlorogenic acid

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Abstract

Ethnopharmacological relevance: Cymbopogon citratus (DC) Stapf leaves infusion is used in traditional medicine for the treatment of inflammatory conditions, however little is known about their bio-active compounds.

Aim of the study: Investigate the compounds responsible for anti-inflammatory potential of *Cymbopogon citratus* (Cy) on cytokines production induced by lipopolysaccharide in human and mouse macrophages, and the action mechanisms involved.

Materials and methods: An essential oil-free infusion of Cy was prepared and polyphenol-rich fractions (PFs) were obtained from it by column chromatography. Chlorogenic acid was identified, by HPLC/PDA/ESI-MS^n. The expression of cytokines, namely TNF-α and CCL5, was analyzed by real-time RT-PCR, on lipopolysaccharide-stimulated human macrophages. Activation of nuclear factor-κB, a master regulator of inflammation, was investigated by Western blot and gene reporter assay. Proteasome activity was assessed using a fluorogenic peptide.

Results: Cymbopogon citratus extract and its polyphenols inhibited the cytokine production on human macrophages. This supports the anti-inflammatory activity of Cy polyphenols in physiologically relevant cells. Concerning the effect on the activation of nuclear factor (NF)-κB pathway, the results pointed to an inhibition of LPS-induced NF-κB activation by Cy and PFs. Chlorogenic acid was identified, by HPLC/PDA/ESI-MS^n, as the main phenolic acid of the Cy infusion, and it demonstrated to be, at least in part, responsible by that effect. Additionally, it was verified for the first time, that Cy and PFs inhibited the proteasome activity, a complex that controls NF-κB activation, having CGA a strong contribution.

Conclusions: The results evidenced, for the first time, the anti-inflammatory properties of *Cymbopogon citratus* through proteasome inhibition and, consequently NF-κB pathway and cytokine expression. Additionally, Cy polyphenols, in particular chlorogenic acid, were highlighted as bio-active compounds.

Keywords: Chlorogenic acid; *Cymbopogon citratus*; Cytokines; NF-κB; Polyphenols; Proteasome

Abbreviations: CGA, chlorogenic acid; Cy, *Cymbopogon citratus*; CCL5, chemokine (C-C motif) ligand 5; FF, flavonoid-rich fraction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; IκB, inhibitory protein κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MS, mass spectrometry; NF-κB, nuclear factor-κB; NO, nitric oxide; PAF, phenolic acid-rich fraction; PDA, photodiode array; PFs, polyphenol-rich fractions; TF, tannin-rich fraction; TLC, thin layer chromatography; TNF-α, tumor necrosis factor-α;

1. Introduction

Inflammation is pointed out in preclinical studies as a major mechanism in the pathogenesis of chronic diseases, namely diabetes, hypertension and cancer (Liu and Zeng, 2012; Osborn and Olefsky, 2012; Price et al., 2012). During an inflammatory response, macrophages release several inflammatory mediators, such as cytokines, which expression is regulated by different intracellular signaling pathways (O'Neill, 2006). Inflammatory stimuli can activate the nuclear factor (NF)-κB by signaling events that lead to the phosphorylation of the inhibitory protein κB (IκB) by the IκB kinase (IKK), with subsequent ubiquitination and degradation by ubiquitin-proteasome system (Vitiello et al., 2012), a pivotal complex in inflammation and cancer development (DiDonato et al., 2012). The IκB degradation unmasks the nuclear localization motif of NF-κB, allowing its rapid translocation to the nucleus and the transcription of many inflammatory mediators, like tumor necrosis factor (TNF)-α and chemokine (C-C motif) ligand 5 (CCL5). Once released by the cell, TNF-α elicits several physiological effects of inflammation (Kopf et al., 2010) and CCL5 has significant chemotactic activity for inflammatory cells (Schober, 2008). The NF-κB activation also induces the transcription of inducible nitric oxide synthase (iNOS), leading to the production of nitric oxide (NO), that is a pro-inflammatory mediator. The overproduction of NO contributes to the pathogenesis of septic shock and inflammatory diseases (Zamora et al., 2000; Guzik et al., 2003). Since the overproduction of these pro-inflammatory mediators raises and maintains inflammation, compounds targeting its expression and production through NF-κB and proteasome pathways are good candidates for attenuating inflammation.

Cymbopogon citratus (DC) Stapf (Cy), Poaceae-Gramineae, commonly known as lemongrass, is a spontaneous perennial graminoid, largely distributed in tropical and subtropical countries. In traditional medicine, aqueous extracts of dried leaves are used for the treatment of several inflammation-based pathologies (Shah et al., 2011). Accordingly, we previously demonstrated that Cy and its polyphenols inhibited nitric oxide (NO) production, in dendritic cells and mouse macrophages, through modulation of p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) 1/2 and NF-κB signaling pathways (Figueirinha et al., 2010; Francisco et al., 2011), which evidences the potential of Cy as source of compounds with anti-inflammatory properties.

The present paper aimed to investigate the effect of Cy, as well its polyphenols, on the lipopolysaccharide (LPS)-induced cytokines production, in human macrophages, and the involvement of NF-κB and proteasome in the anti-inflammatory profile of *Cymbopogon citratus*. Considering the potential of phenolic acids to the anti-inflammatory properties of Cy, HPLC/PDA/ESI-MS^n was performed in order to identify the main phenolic acid present, its biological activity being subsequently assessed on the pure compound.

2. Materials and Methods

2.1. Plant material, infusion preparation and extract fractionation

Dry leaves of *Cymbopogon citratus* Stapf (Cy) were purchased from ERVITAL® (Mezio, Castro Daire, Portugal). The plant was cultivated in the region of Mezio, Castro D´Aire (Portugal). A voucher specimen was deposited in the Herbarium of Aromatic and Medicinal Plants of the Faculty of Pharmacy - University of Coimbra (A. Figueirinha 0109). The identity of the plant was confirmed by J. Paiva (Botany Department, University of Coimbra, Portugal). An lipid- and essential oil-free infusion was prepared and fractionated by column chromatography as previously described (Figueirinha et al., 2008). 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 *μ*m), 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 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 Cy extract and the polyphenol-rich fractions were weighted in sterilized and humidity-controlled conditions, and then Cy extract solubilized in sterilized water and polyphenol-rich fractions in sterilized phosphate buffered saline.

2.2. HPLC and mass spectrometry analyses

Structural elucidation of chlorogenic acid (CGA) was carried out on a Surveyor liquid chromatograph equipped with a photodiode array (PDA) detector (Surveyor) and interfaced with a Finnigan LCQ Advantage Ion Max tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an API-ES ionization chamber. Separation was performed on a Spherisorb ODS-2 column (150x2.1 mm i.d.; particle size, 3 μm; Waters Corp., Milford, MA, USA) and a Spherisorb ODS-2 guard cartridge (10x4.6 mm i.d.; particle size, 5 μm; Waters Corp., Milford, MA, USA) at 25ºC. A mobile phase constituted by 2% aqueous formic acid (v/v) (A) and methanol (B) was used with a discontinuous gradient of 5–15% B (0–10 minutes), 15–25% B (10–15 minutes), 25–50% B (15–40 minutes), 50–80% B (40–50 minutes), followed by an isocratic elution (50–60 minutes), a gradient 80–100% B (60–65 min) and other isocratic elution for 5 minutes, at a flow rate of 200 μL.min^{-1}. The first detection was done with a PDA detector in a wavelength range 200–400 nm, followed by a second detection in the mass spectrometer. Mass analyses were obtained in the negative ion mode. The mass spectrometer was programmed to perform three consecutive scans: full mass (*m/z* 125–1500), MS^2 of the most abundant ion in the full mass and MS^3 of the most abundant ion in the MS^2. Source voltage was 4.5 kV and the capillary voltage and temperature were -10 V and 250ºC, respectively. Nitrogen was used as sheath and auxiliary gas at 20 Finnigan arbitrary units. The normalized energy of collision was 45%, using helium as collision gas. Data treatment was carried out with XCALIBUR software (Thermo Scientific, Waltham, MA, USA).

HPLC profiles of the Cy extract and PAF, as well the CGA quantification were performed in a chromatograph equipped with a PDA (Gilson Electronics SA, Villiers le Bel, France). The studies were carried out as previously (Figueirinha et al., 2008). Chromatographic profiles were acquired in the wavelength range of 200–600 nm and recorded at 280 and 320 nm. Data treatment was carried out with Unipoint®, version 2.10 software (Gilson, Middleton, WI, USA).

A standard stock solution of chlorogenic acid (HPLC-grade purity from Sigma) was used for the quantification. Calibration curve was obtained by diluting stock standard in methanol to yield 0.3-10 μg/mL. The absorbance was recorded at 320 nm and the linearity between the response and concentration was evaluated by regression analysis. The samples were analyzed in triplicate.

2.3. Cell culture and chemical treatment

Human monocytes were isolated from buffy coats of healthy volunteer donors by centrifugation into Ficoll cushions and adherence to plastic dishes. Adhered monocytes were cultured in RPMI (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine, 40 μg/mL of gentamicin (Lonza, Basel, Switzerland) and 5% (v/v) heat-inactivated human serum for 2 weeks in the absence of exogenous cytokine mixtures in order to differentiate into macrophages. RAW 264.7 (ATCC number: TIB-71) was cultured in Iscove´s Modified Dulbecco's Media (Sigma–Aldrich Química, Madrid, Spain) supplemented with 10% (v/v) non-inactivated fetal bovine serum (Gibco, Paisley, UK), 100 *U*/mL penicillin and 100 μg/mL streptomycin (both from Sigma–Aldrich Química, Madrid, Spain). The cells were cultured at 37°C in a humified atmosphere of 95% air and 5% CO_2. The investigation conforms to the principles outlined in the Declaration of Helsinki.

For the experiments, cells were maintained in culture medium (control) or pre-incubated with 1.115 mg/mL Cy, 530 μg/mL PAF, 97.5 μg/mL FF, 78 μg/mL TF, or indicated concentrations of CGA (Sigma Chemical Co., St. Louis, MO, USA) for 1 h. Then, 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6) (Sigma Chemical Co., St. Louis, MO, USA) was added for the indicated period of time. The Cy concentration used was based in previous studies (Figueirinha et al., 2010; Francisco et al., 2011) while the PFs concentration was based on their ratios in the Cy extract after fractionation: PAF (23.8%), FF

(4.4%) and TF (3.5%). The CGA concentrations were selected based on its concentration in PAF (3.33%) and PAF ratio in the Cy extract (23.8%).

2.4. RNA extraction and real-time RT-PCR

Human macrophages were pre-incubated with Cy extract or PFs for 1h and then, with LPS for 8h. Total RNA was isolated from cells with Trizol® reagent (Invitrogen, Barcelona, Spain). The concentration and purity of the RNA samples were evaluated by spectrophotometry using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). RNA reverse transcription was performed using iScriptTM select cDNA synthesis kit (BioRad, Hercules, CA, USA), accordingly to manufactory instructions, on C1000^{TM} Thermal Cycler (BioRad, Hercules, CA, USA).

The resulting cDNA of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as endogenous control, TNF-α and CCL5 were amplified by real-time RT-PCR, using the SYBR-Green (BioRad, Hercules, CA, USA) assay to monitor the amplification reactions on a Bio-Rad My Cycler iQ5. For that, specific primers (MWG Biotech, Ebersberg, Germany) were designed using Beacon Designer® Software v7.2 (Primier Biosoft International) (Table 1). Gene expression changes were analyzed using the built-in iQ5Optical system software v2, with the Pfaffl method (Pfaffl, 2001). Gene expression was expressed as relative fold changes compared to LPS and normalized to GAPDH.

2.5. Western blot

Total cell lysates were prepared using the RIPA buffer [50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS and 2 mM EDTA] freshly supplemented with 1 mM DTT, protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Cytoplasmic and nuclear extracts were obtained by a commercial nuclear extract kit (Active Motif, Rixensart, Belgium), accordingly to manufactory instructions. Protein concentration of cell lysates was determined by the bicinchoninic acid protein assay. Cell lysates were denaturated at 95 ºC, for 10 min, in sample buffer [0.125 mM Tris (pH 6.8), 2% (w/v) SDS, 100 mM DTT, 10% glycerol and bromophenol blue].

Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis transferred to polyvinylidene fluoride membranes and specific antibodies (Cell Signaling Technologies, Danvers, MA, USA) against phospho-IκBα, total IκBα and NF-κB p65 were used. The immune complexes were detected using the enhanced chemifluorescence reagent (GE Healthcare, Chalfont St. Giles, UK) on the Storm 860 (GE Healthcare) and analyzed by software ImageQuant TL®. To demonstrate equivalent protein loading, membranes were stripped and reprobed with antibodies against actin (Millipore, Bedford, MA, USA) or lamin (Calbiochem, Darmstadt, Germany).

2.6. Dual-Luciferase assay

RAW 264.7 were transiently transfected with NF-κB-dependent firefly luciferase-expressing plasmid using Lipofectamine™ LTX and Plus Reagent (Invitrogen, Paisley, UK). After chemical treatment and incubation with LPS for 8h, the luciferase activity was measured using the Dual Luciferase® reporter assay system (Promega, Madison, WI, USA), accordingly to manufacturer's instructions, in the MicroLumat Plus LB96V Luminometer (EG&G Berthold, Bad Wildbad, Germany). Injectors were programmed to dispense 50 μL of LAR II and Stop & Glo® reagent and measure was performed using 2-second delay and a 10-second read time.

2.7. Chymotrypsin-like activity of proteasome

Cytosolic extracts were prepared using lysis buffer [50 mM Tris-HCl (pH 7.6) with 1 mM DTT] followed by sonication and centrifugation to remove cell debris. Fluorogenic peptide Suc-LLVY-AMC (Biomol International, Plymouth Meeting, PA, USA) at 70 μM was added to 20 μg protein of cytosolic extracts. Fluorescence was measured at 37ºC in Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA), using excitation wavelength of 360 nm and emission wavelength of 460 nm. The Gen 5 software (Biotek, Winooski, VT, USA) was used to monitor the results.

2.8. Nitric oxide production

The production of NO was measured by the accumulation of nitrites in the culture supernatants, using a colorimetric reaction with the Griess reagent, as previously described (Francisco et al., 2011).

2.9. Statistical analysis

Two-sided unpaired t-test was used to compare LPS-stimulated cells with control, while One-way ANOVA followed by Dunnett's test was applied to compare the effect of different treatments on LPS-stimulated cells. GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analysis. 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. Chlorogenic acid identification and quantification

The main phenolic acid from Cy was separated and identified by HPLC/PDA/ESI-MS^n. UV spectrum showed the typical maxima of a caffeic acid derivative: 251, 298sh and 326 nm, mass spectrum at MS^1 being characterized for the presence of two main signals: the base peak at *m/z* 353 (100%) and a slightly less abundant peak at *m/z* 707 (79%) (Fig. 1A). The ion at *m/z* 353 is consistent with the presence of a compound with a $C_{16}H_{18}O_9$ formula, characteristic of a caffeoylquinic-type phenolic acid (Fang et al., 2002). As in other quinic acid esters, the caffeoylquinic acids in the negative ion mode originate ions by two competing pathways (Fig. 1B); pathway I, with the ions Q_2 (*m/z* 173), C_1 (*m/z* 179) and C_2 (*m/z* 135) and pathway II, with the characteristic presence of the fragment Q_1 (*m/z* 191) (Bravo et al., 2007). For this compound, the presence of fragments at *m/z* 191 (100%) (MS^2) and *m/z* 173 (100%) (MS^3) seems to confirm the presence of a caffeoylquinic acid. The relative abundance of fragments referred can be used for the identification of the chlorogenic acids: cryptochlorogenic, chlorogenic and neochlorogenic acids (Fang et al., 2002). The fragmentation of cryptochlorogenic acid follows pathway I, presenting the ion Q_2 at *m/z* 173 as the base peak of the MS^2 spectrum, while chlorogenic and neochlorogenic acids follow pathway II, with a base

peak at *m/z* 191, corresponding to fragment Q_1. In case of chlorogenic acid (CGA), the pseudo molecular ion originates a fragment Q_1 at *m/z* 179 with a relative abundance lower (about 5%) than that of the neochlorogenic acid (20-60%). For the main phenolic acid of Cy we verified that the fragment at *m/z* 179 presented a relative abundance of 5% (Fig. 1A), suggesting the presence of CGA as the most probable structure. The signal at *m/z* 707 was reported as a result of a dimeric adduct of the pseudo-molecular ion *m/z* 353, in the same analytical conditions (Bravo et al., 2007).

Two main phenolic acids, the CGA and a *p*-coumaric acid derivative, with retention times of 19.95 and 25.33 min, respectively, were detected in a phenolic acid-rich fraction (PAF) prepared from Cy extract, CGA being the most representative (Fig. 1C). The CGA quantification by HPLC-PDA system revealed that this phenolic acid represents 1.11% (w/w) of Cy extract and 3.33% (w/w) of PAF.

3.2. Pro-inflammatory cytokines expression

Given the important role of cytokines production in inflammation, the expression of TNF-α and CCL5 in LPS-stimulated human macrophages were evaluated by real-time RT-PCR (Fig. 2A and 2B). The pre-treatment of cells with Cy extract decreased the LPS-induced TNF-α mRNA levels by 64.89±5.04%. In addition, PFs reduced the LPS-induced TNF-α expression, being phenolic acids and tannins as potent as Cy extract. Relatively to CCL5 expression, Cy extract inhibited LPS-induced CCL5 mRNA levels by 47.04±12.52. Cy polyphenols showed some inhibition of CCL5 expression, however it was not statistically significant. Taken together, data evidence the pharmacological importance of *Cymbopogon citratus* and its polyphenols through the inhibition of cytokines expression.

3.3. NF-κB activation

The expression of pro-inflammatory cytokines is mainly regulated by NF-κB pathway. So, the effect of Cy extract and its PFs on NF-κB activation was assessed by western blot and reporter assays. The pre-treatment of human macrophages with Cy extract, maintained the LPS-induced IκBα phosphorylation (Fig. 2C) and blocked the degradation of IκBα (Fig. 2D),

suggesting the inhibition of NF-κB activation. Additionally, the inhibition of IκBα degradation by TF was verified, suggesting the TF contribution to the Cy inhibitory activity.

Reinforcing these results, the same behavior was confirmed in murine macrophages. In fact, pre-treatment with Cy extract maintained the phosphorylation levels of IκBα (Fig. 3A) and the blockade of IκBα degradation induced by LPS (Fig. 3B). It was verified that none of the three fractions have a significant effect in LPS-induced IκBα phosphorylation, but PAF and FF inhibited the IκBα degradation (Fig. 3B). Interestingly, a standard of the main phenolic acid in Cy extract, the chlorogenic acid, maintained the phosphorylation levels of IκBα, such as the Cy extract. Immunodetection of p65 in cytosolic and nuclear extracts demonstrated the inhibition of p65 translocation to the nucleus by Cy extract (Figs. 3C and 3D), this result being consistent with a NF-κB inhibition by Cy. Additionally, all the PFs inhibited the transcriptional activity of NF-κB (Fig. 3E), thus suggesting their contribution to the Cy extract anti-inflammatory potential by inhibition of NF-κB activation.

All together, the data demonstrate that Cy extract inhibited the NF-κB activation in human and murine macrophages, being polyphenols partially responsible by this anti-inflammatory mechanism.

3.4. Proteasome activity

The ubiquitin-proteasome system has a central role in the regulation of NF-κB as well other inflammatory signaling pathways (Shen et al., 2006). So, the chymotrypsin-like activity of proteasome was investigated. In LPS-activated murine macrophages, Cy extract significantly decreased the proteasome activity by 38±6.4% (Fig. 4), which is concordant with the blockade of IκBα degradation and maintenance of its phosphorylation status. Both phenolic acid- and tannin-rich fractions inhibited proteasome activity; ~~However, only~~ in particular, PAF decreased~~, with statistic significance,~~ the proteasome activity by 24.5±9.8%, suggesting a ~~specific effect~~ strong contribution of Cy phenolic acids for this activity. Therefore, the effect of chlorogenic acid, the main phenolic acid present in Cy extract and PAF, was assessed. CGA (17.5 μg/mL) inhibited the proteasome activity by 28.43±22.09% and, so, strongly contributes to the inhibition of proteasome by Cy in LPS-stimulated murine macrophages. This data also supports the anti-

inflammatory properties of Cy extract through inhibition of NF-κB activation and, consequently, cytokines expression.

3.5. Nitric oxide production

Given the inhibitory activity of CGA on NF-κB pathway and proteasome activity, its anti-inflammatory properties were then evaluated through measurement of NO production by Griess assay, in murine macrophages. The inhibition of LPS-induced nitrite production by CGA is statistically significant at 140 μg/mL (Fig. 5), which indicates that CGA inhibited proteasome activity at lower doses (17.5 μg/mL) but a higher concentration is needed to inhibit the production of pro-inflammatory mediators, such as NO. None of the concentrations used affected the cell viability (data not shown).

4. Discussion

In this study, the anti-inflammatory action of a lipid- and essential oil-free extract from *Cymbopogon citratus,* in human macrophages, was proved. The results demonstrated that Cy extract inhibits cytokine expression through NF-κB pathway and, for the first time, this inhibition through ubiquitin-proteasome system was referred. Moreover, data support a contribution of polyphenols for the anti-inflammatory activity of the Cy extract, pointing chlorogenic acid as a bioactive compound responsible for proteasome system inhibition.

Cytokines have a central role in inflammation, driving the inflammatory response to protective immunity or to induction of immunopathology, with the clinical outcome partly determined by the balance between pro- and anti-inflammatory molecules. Here a decrease in LPS-induced TNF-α and CCL5 expression by Cy extract, in human macrophages, was verified. TNF-α is a cytokine that induces several pro-inflammatory effects (Kopf et al., 2010) and with an important role in linking inflammation and cancer (Liu and Zeng, 2012), while CCL5 is a chemokine that recruits leukocytes, including T-cells and monocytes (Schober, 2008). Since the deregulated production of these cytokines was associated with inflammatory and autoimmune

diseases, the inhibition of both TNF-α and CCL5 expression by Cy extract evidenced its anti-inflammatory potential.

Elucidating the bioactive compounds of Cy, the present data demonstrated that Cy polyphenols could account, at least partially, for the inhibition of TNF-α expression. The main flavonoids present in Cy are luteolin derivatives (Figueirinha et al., 2008), and previous results evidenced luteolin as the best flavonoid candidate to provide anti-inflammatory relief *in vivo* (Comalada et al., 2006) as well as to inhibit the LPS-induced cytokine production through inhibition of the NF-κB pathway (Chen et al., 2007). NF-κB activation by an inflammatory stimulus, like LPS, is known to induce the expression of several inflammatory enzymes and mediators, such as iNOS, NO and cytokines (Vitiello et al., 2012). Here, it was evidenced that the anti-inflammatory effect of Cy extract is, at least in part, mediated by the NF-κB pathway inhibition. Consistent with this mechanism, we previously described the decrease in NF-κB-dependent NO production by Cy and its PFs in murine macrophages (Francisco et al., 2011). The TNF-α biosynthesis could be regulated not only by NF-κB pathway but also by p38 MAPK (Xie et al., 2012) and JNK (Kang et al., 2010). Noteworthy, we have previously found that Cy inhibited p38 MAPK and JNK activation, indicating that both NF-κB and MAPK could be involved in the inhibition of LPS-induced TNF-α production by Cy extract. Supporting our data, Cy extract also posses *p*-coumaric derivatives that were previously described as inhibitors of NF-κB-dependent iNOS and COX-2 expression (Yen et al., 2008).

The inhibition of ubiquitin-proteasome system, with consequent blockade of NF-κB pathway, reveals the anti-inflammatory potential of Cy extract. In fact, proteasome regulates protein degradation and homeostasis, having a key role in key inflammatory signaling pathways (Shen et al., 2006), cell cycle arrest and apoptosis. Therefore, ubiquitin-proteasome inhibition has been pointed not only as an anti-inflammatory target but also as an anti-neoplasic one (Gräwert and Groll, 2012). The available proteasome inhibitors, like bortezomib, are effective and selective, even though they possess toxic effects (Gräwert and Groll, 2012). Therefore, Cy extract could provide a potent but less cytotoxic proteasome inhibitor and, consequently, be a source of new anti-inflammatory and anti-neoplasic drugs.

The phenolic acid- and tannin-rich fractions from Cy, demonstrated proteasome inhibitory activity, evidencing the presence of active compounds. Accordingly, tannins were reported to

modulate proteins involved in ubiquitin-proteasome system (Li et al., 2008). Chemical characterization of Cy extract showed the chlorogenic acid as the main phenolic acid. Curiously, significant proteasome inhibitory properties, similar to that of *Cymbopogon citratus* extract, were evidenced when a standard of CGA was assayed for the concentration occurred in the PAF (17.5 *μ*g/mL). However, 1.115 mg/mL Cy, which only contains 12.37 μg/mL of CGA, have a higher effect on proteasome activity than 17.5 μg/mL CGA, suggesting that non-phenolic acid compounds were also involved. ~~In fact,~~ CGA was previously described as a proteasome inhibitor and anti-neoplasic agent (Cichocki et al., 2010), as well as a NF-κB inhibitor (Shan et al., 2009), which reinforces our results. Accordingly, our data demonstrated that CGA maintains the phosphorylation levels of IκBα, which evidences an inhibition of both proteasome activity and NF-κB activation. Since NF-κB pathway controls the production of cytokines and NO, the anti-inflammatory properties of CGA were also investigated. The results evidenced an inhibition of LPS-induced NO production by CGA but at higher concentrations than those required for proteasome and NF-κB inhibition, which indicates that CGA strongly contributed to inhibition of proteasome activity by Cy, but other compounds, namely tannins and flavonoids, have an higher contribution to the Cy anti-inflammatory properties. Therefore, CGA could be pointed as a bioactive compound of Cy and a good candidate for further research as inhibitor of ubiquitin-proteasome system and therapeutical agent in diseases associated with proteasome deregulation, such as cancer.

In conclusion, this work better elucidates the anti-inflammatory mechanism of *Cymbopogon citratus* via the inhibition of proteasome activity and, consequently, NF-κB pathway and cytokine expression. Additionally, phenolic compounds, in particular chlorogenic acid, were pointed as bio-active compounds, revealing the importance of *Cymbopogon citratus* as source of new anti-inflammatory natural drugs.

Conflict of interest

No conflict to disclose.

Acknowledgements

We thank Dr. O. Vieira for the kind gift of the mouse macrophage-like cell line RAW 264.7, to Ervital® for the provision of the plant, to Dr. J. Paiva for the plant classification and to Laboratory of Mass Spectrometry of CEF/UC node, integrated in the National Mass Spectrometry Network (RNEM) of Portugal, for the mass spectra. Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain) is thanked for providing buffy coats. We are also grateful to Cristina Gómez, Isabel Fernández-Pisonero, Patricia Maeso Hernández and Fátima Nunes for their technical assistance.

This work was supported by FEDER/COMPETE (FCOMP-01-0124-FEDER-011096) and Foundation for Science and Technology (FCT), by the projects PTDC/SAU-FCF/105429/2008, PEst-OE/SAU/UI0177/2011 and PEst-C/SAU/LA0001/2011, and PhD fellowships SFRH/BD/46281/2008 and SFRH/BD/30563/2006, and also by a project from the Spanish Ministry of Science SAF09/08692.

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Table 1. Oligonucleotide primer pairs used for real-time RT-PCR

Gene name	Primer sequences (5´-3´)
GAPDH	F:ACAGTCAGCCGCATCTTC
	R:GCCCAATACGACCAAATCC
TNF-α	F:AGAAGACCTCACCTAGAA
	R:TCTCAAGGAAGTCTGGAA
CCL5	F:CAGTGAGCTGAGATTGTG
	R:TTTGTTGTTGTTGTTGTGA

F: Forward sequence; R: Reverse sequence.

Figure legends

Fig. 1. Chlorogenic acid of *Cymbopogon citratus* leaves. (A) Mass spectra. (B) Fragmentation pattern for hydroxycinnamic esters of quinic acid. (C) HPLC profile from phenolic acid-rich fraction, recorded at 320 nm, illustrating two main phenolic acids, namely chlorogenic acid (Rt=19.95 min) and *p*-coumaric acid (Rt=25.33 min), on conditions previously reported (Figueirinha et al., 2008).

Fig. 2. *Cymbopogon citratus* (Cy) and its polyphenol-rich fractions inhibited LPS-induced TNF-α (A) and CCL-5 (B) production as well as NF-κB activation (C and D), in human macrophages. In the graphics, the results were expressed as fold changes relatively to LPS and each value

represents the mean ± SEM from 3 independent experiments performed in duplicate ($^{###}p<0.001$, when compared to control; *p<0.05 and ***p<0.001, when compared to LPS). The NF-κB activation was analyzed by western blot using antibodies against phospho-IκBα (C) and total IκBα (D). Each blot shown is representative of 3 blots yielding similar results.

Fig. 3. *Cymbopogon citratus* (Cy) and its polyphenol-rich fractions inhibited LPS-induced NF-κB activation, in murine macrophages. Total cell extracts were analyzed by western blot using antibodies against phospho-IκBα (A) and total IκBα (B). The migration of NF-κB p65 to the nucleus was analyzed using cytoplasmic (C) and nuclear (D) extracts. Each blot shown is representative of 3 blots yielding similar results. The NF-κB transcriptional activity was measured using a NF-κB-dependent luciferase reporter plasmid. The results were expressed as fold changes relatively to LPS and each value represents the mean ± SEM from 3 independent experiments (*p<0.05, when compared to LPS).

Fig. 4. *Cymbopogon citratus* (Cy), phenolic acid-rich fraction (PAF) and chlorogenic acid (CGA) inhibited the chymotrypsin-like activity of murine macrophage proteasome. The results were expressed as fold changes relatively to LPS and each value represents the mean ± SEM from 3 independent experiments (*p<0.05 and **p<0.01, when compared to LPS).

Fig. 5. Chlorogenic acid (CGA) inhibited the LPS-induced nitrite production, in murine macrophages. The results were expressed as fold changes relatively to LPS and each value represents the mean ± SEM from 3 independent experiments (*p<0.05, when compared to LPS).

Graphical abstract

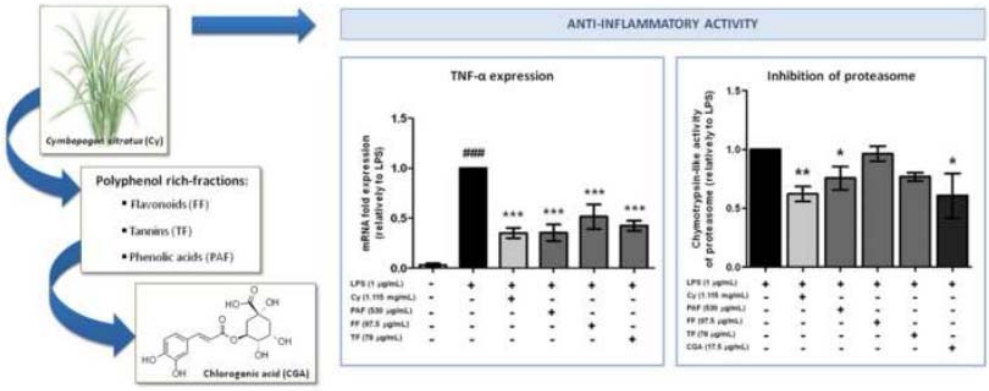


Figure 1

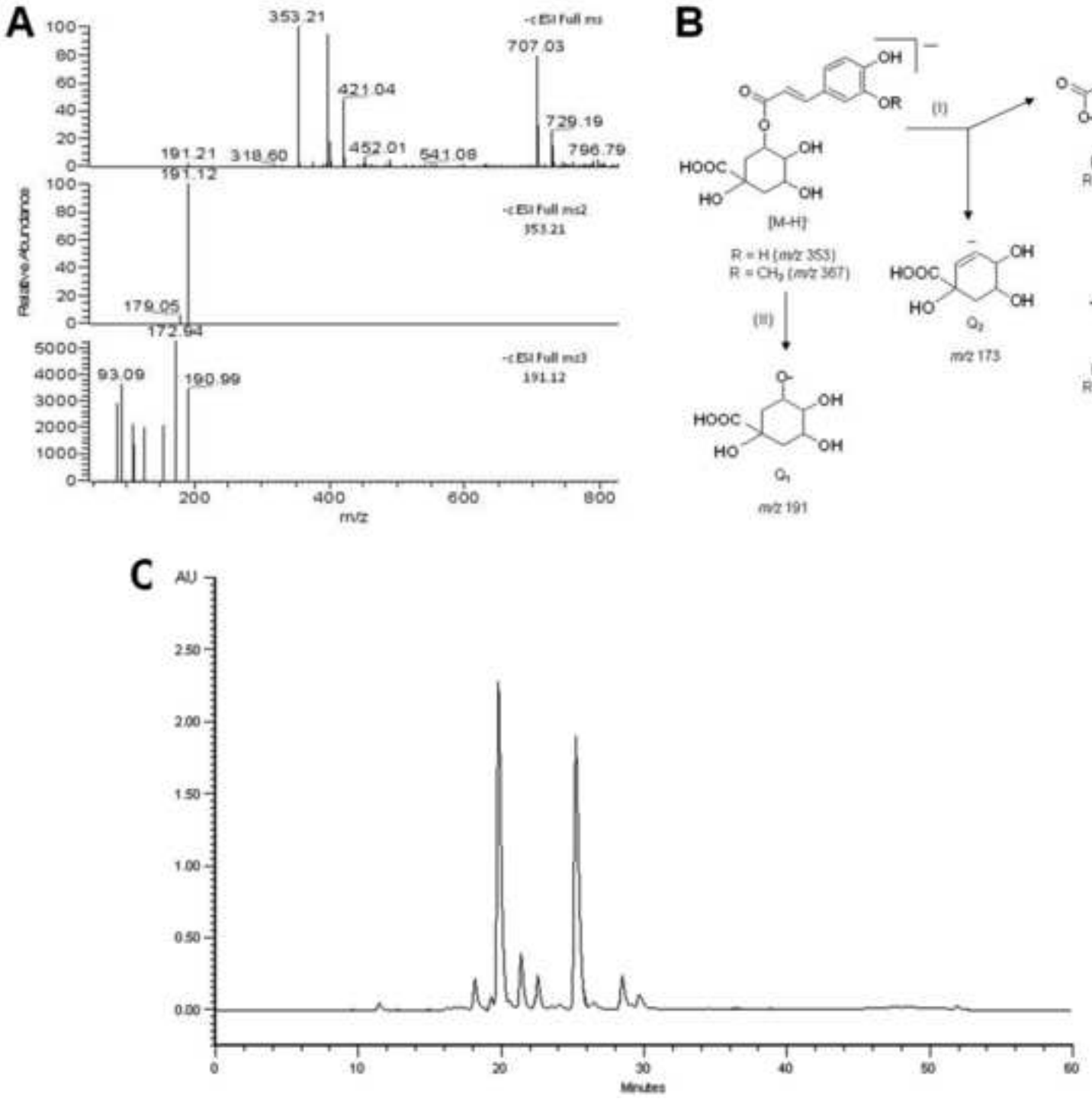


Figure 2

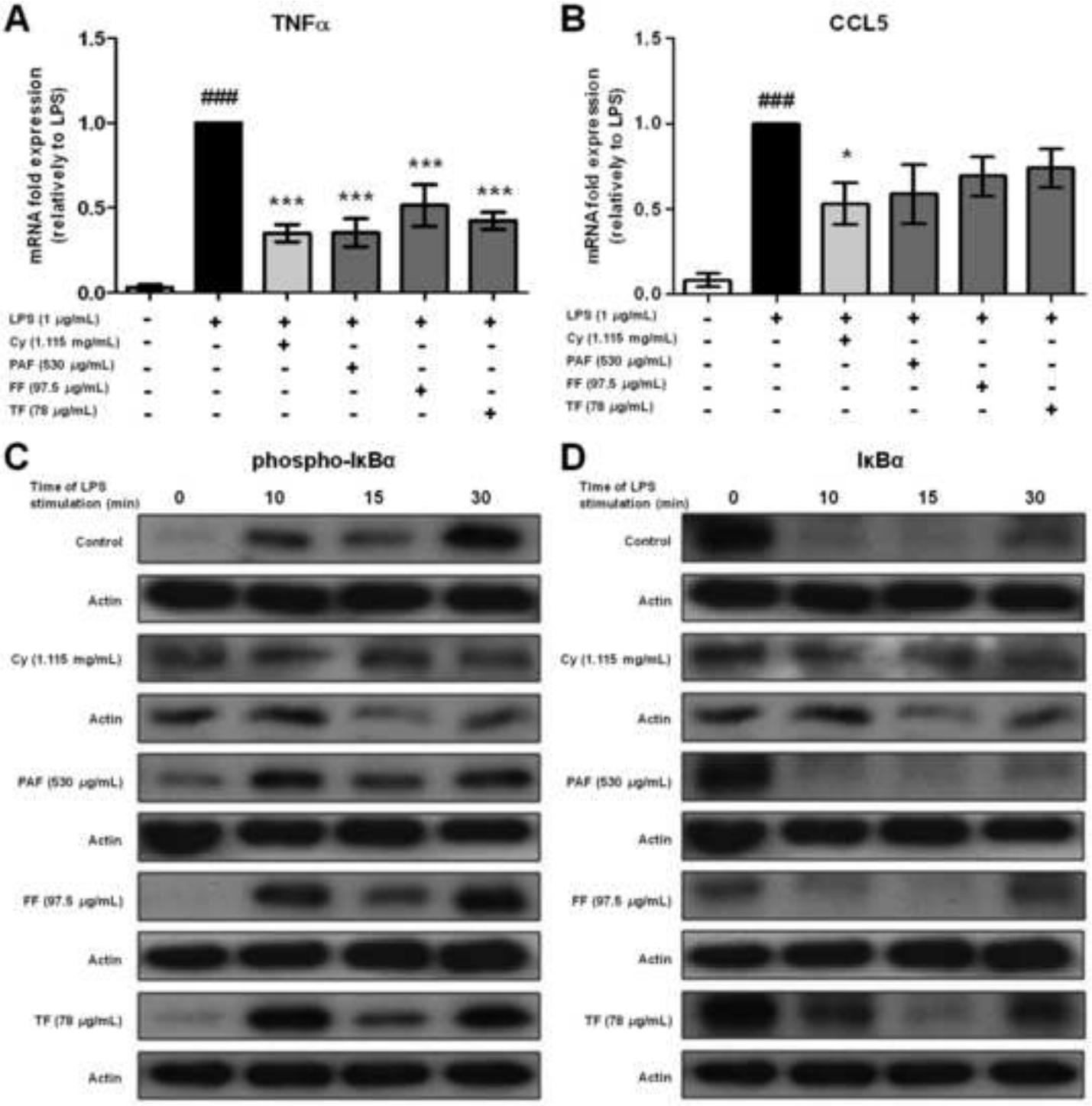
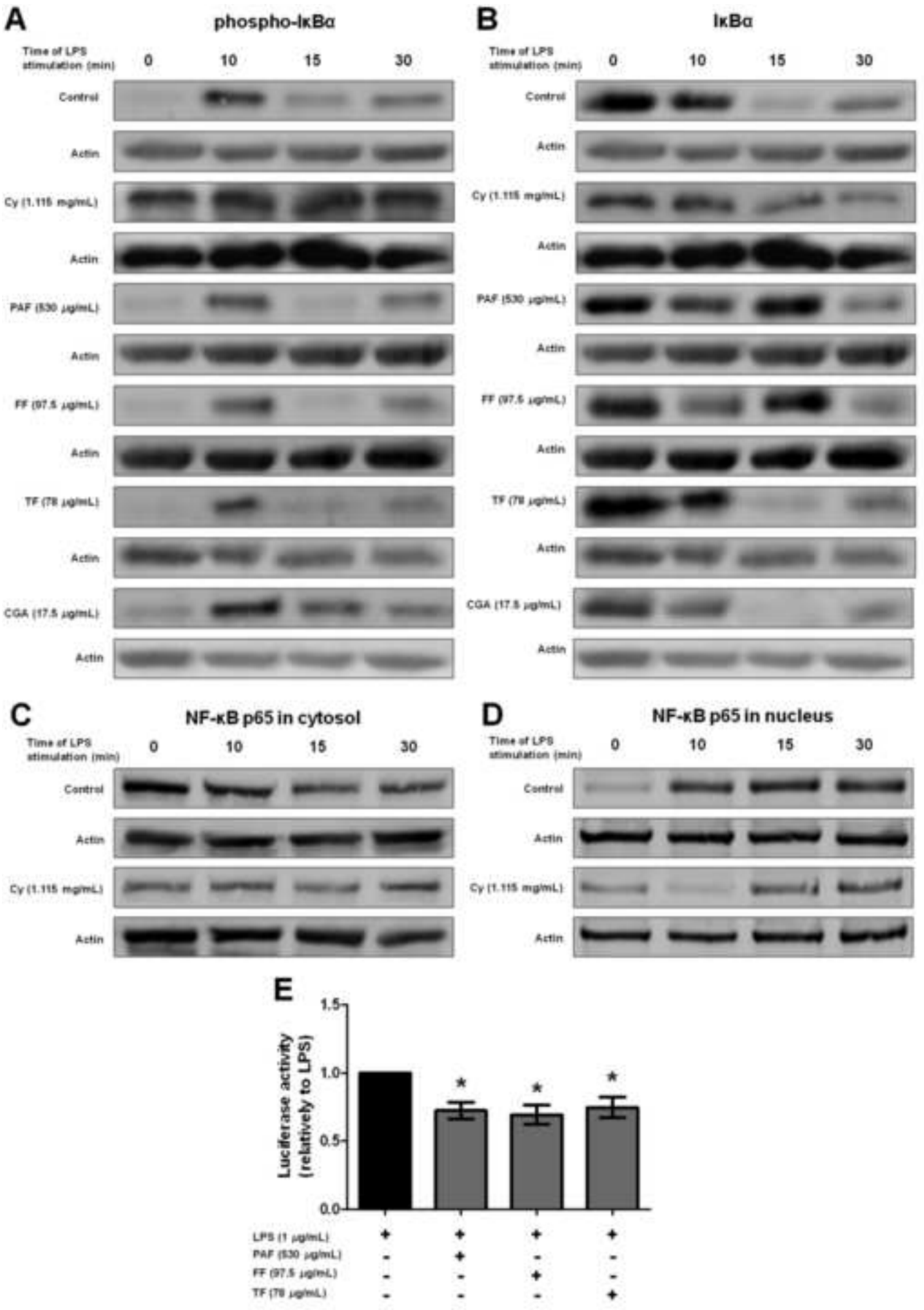


Figure 3



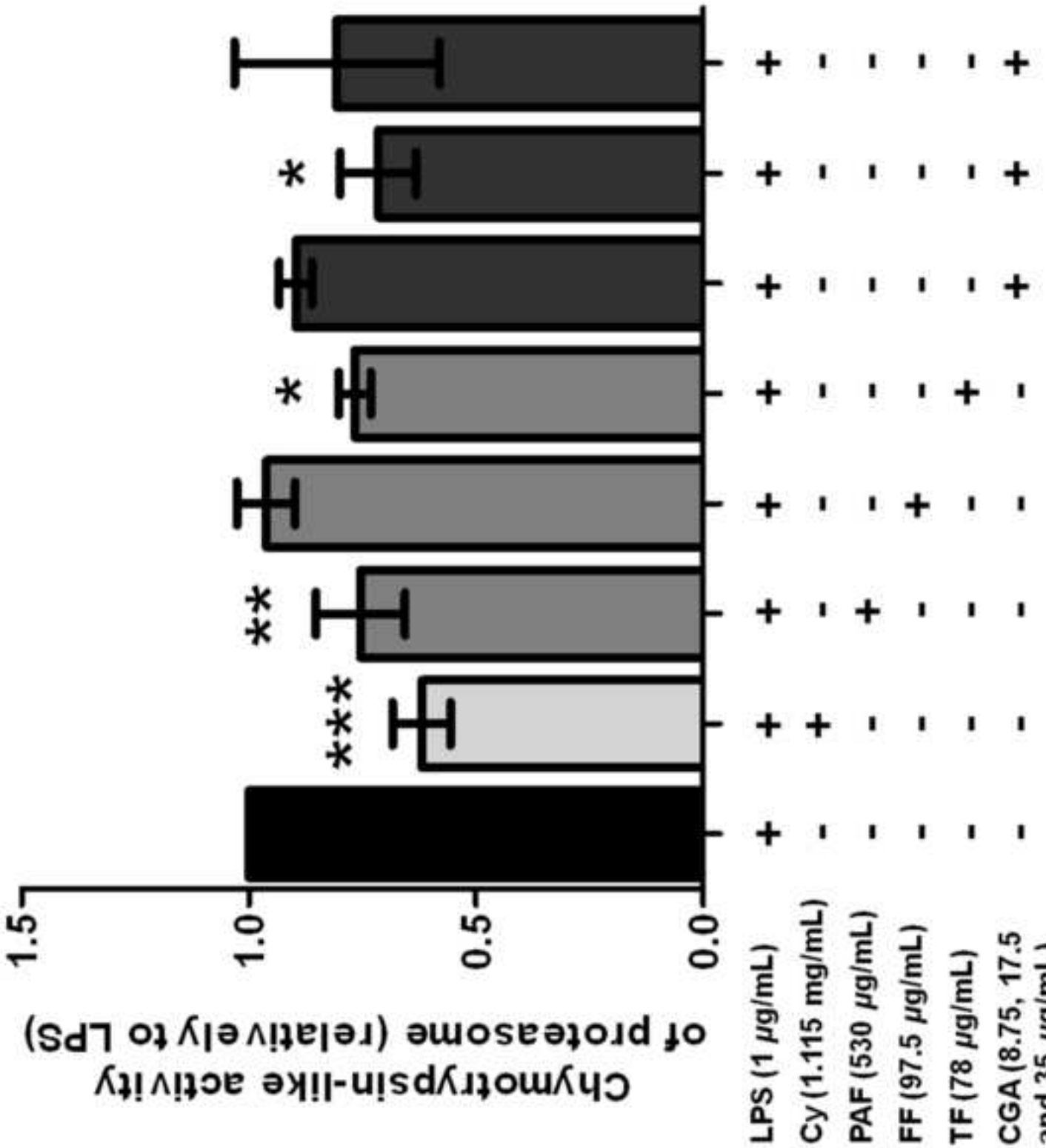


Figure 4_Revised

Figure 5_Revised