Vera Lúcia Gomes Francisco

Drug discovery of new anti-inflammatory phytochemicals using *in vitro* bio-guided assays: The case study of *Cymbopogon citratus*

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Drug discovery of new anti-inflammatory phytochemicals using *in vitro* bio-guided assays: The case study of *Cymbopogon citratus*

Vera Lúcia Gomes Francisco

A thesis presented to the Faculty of Pharmacy of the University of Coimbra in fulfillment of the requirements for the degree of Doctor in Sciences and Health Technologies (specialty in Cellular and Molecular Biology)

Front cover: *Cymbopogon citratus* (DC.) Stapf (original image kindly supplied by Mónica Zuzarte) and luteolin 7-*O*-β-glucopiranoside structure. Image by Dora Gomes Francisco.

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Aos meus pais

In vitro cognitio sed in vivo veritas

(Richard Gryglewski)

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Publications and communications

This PhD thesis includes the work presented in the following publications and communications.

Papers published in international scientific periodicals with referees:

Francisco V., Costa G., Figueirinha A., Marques C., Pereira P., Neves B.M., Lopes M.C., García-Rodríguez C., Cruz M.T., Batista M.T., 2013. Anti-inflammatory activity of *Cymbopogon citratus* leaves infusion via proteasome and nuclear factor-kB pathway inhibition: contribution of chlorogenic acid. Journal of Ethnopharmacology 148, 126-34.

Costa G.*, **Francisco V.***, Lopes M.C., Cruz M.T., Batista M.T., 2012. Intracellular signaling pathways modulated by phenolic compounds: application for new anti-inflammatory drugs discovery. Current Medicinal Chemistry 19, 2876-900.

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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, 818-27.

Papers submitted in international scientific periodicals with referees:

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Abstracts published in international scientific periodicals with referees

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Francisco V., Figueirinha A., Neves B.M., García-Rodríguez C., Lopes M.C., Cruz M.T. & Batista M.T., 2010. Polyphenols from *Cymbopogon citratus* inhibit iNOS expression and NO production - a promising source of new anti-inflammatory drugs. Planta Medica 76, 1249.

Francisco V., Figueirinha A., Neves B.M., García-Rodríguez C., Lopes M.C., Cruz M.T. & Batista M.T., 2010. Inhibition of NF-kB activation by *Cymbopogon citratus* leaves and their polyphenols in human macrophages: a potential natural source of anti-inflammatory drugs. Basic and Clinical Pharmacology and Toxicology 107 Suppl 1, 286.

Francisco V., Cruz M.T., Figueirinha A., Marques C., Pereira P., Neves B.M., Lopes M.C., Batista M.T., 2009. Inhibition of proteasome activity by *Cymbopogon citratus* leaves: potential natural source of anti-cancer and anti-inflammatory drugs. Revista de Fitoterapia 9 Suppl 1.

Francisco V., Cruz M.T., Figueirinha A., Neves B.M., Lopes C., Batista M.T., 2009. Anti-inflammatory activity and action mechanism of *Cymbopogon citratus* leaves in lipopolysaccharide-stimulated RAW 264.7 macrophages. European Journal of Immunology 39 Suppl 1.

Francisco V., Cruz M.T., Figueirinha A., Neves B.M., Lopes C., Batista M.T., 2009. Inhibition of LPS-induced nuclear factor NF-kB activation by *Cymbopogon citratus* leaves in macrophages: a strategy to develop new anti-inflammatory drugs. Planta Medica 75.

Oral communications:

Discovery of new anti-inflammatory drugs from medicinal plants using bio-guided assays. Seminar at the Center for Neurosciences and Cell Biology. Coimbra, 11st January 2013.

A bio-guided assay reveals *Cymbopogon citratus* as source of new anti-inflammatory drugs. XLII Anual meeting of the Portuguese Pharmacological Society. Lisbon, 2nd February, 2012.

Molecular mechanisms behind the therapeutic properties of *Cymbopogon citratus*: effect on inflammatory mediators and proteasome activity. 2nd ScienCEF. Coimbra, 12th July, 2011.

Communications as poster:

Francisco V., Costa G., Figueirinha A., Liberal J., Lopes M.C., García-Rodríguez C., Cruz M.T., Batista M.T.. Screening of new plant-derived anti-inflammatory compounds using *in vitro* studies: potential of *Cymbopogon citratus*. CEF´s Meeting. Coimbra, 25th September, 2012.

Costa G., **Francisco V.**, Cruz M.T., Lopes M.C., Batista M.T.. Inhibition of proteasome system by natural compounds: source of new anti-inflammatory and anti-cancer drugs. 6th European Congress of Pharmacology. Granada-Spain, 2012.

Francisco V., Figueirinha A., Costa G., Marques C., Pereira P., Lopes M.C., García-Rodríguez C., Cruz M.T., Batista M.T.. Anti-inflammatory and anti-cancer potential of *Cymbopogon citratus* and their polyphenols in human macrophages. 3rd CEF´s Meeting. Coimbra, 27th September, 2011.

Abstract

Inflammation is a complex immunological process involved in protection against infection or injury tissue and homeostasis maintenance that when dysregulated leads to a chronic inflammation state and disease, including Alzheimer, diabetes, cardiovascular diseases, atherosclerosis and cancer. However, given that the current anti-inflammatory drugs are not totally safe and effective; there is the need to develop new anti-inflammatory agents. The inflammatory response comprises inducers, being a classical one the bacterial lipopolysaccharide (LPS), the sensors expressed in the inflammatory cells, signaling transduction pathways that induce the expression of inflammatory genes, and effectors. Macrophages are key inflammatory cells that provide an innate immune defense against foreign agents and also promote an adaptative immune response by the secretion of several inflammatory mediators, which expression is controlled by intracellular signaling pathways. Therefore, compounds targeting signal transduction pathways and mediators produced by macrophages stimulated with an inflammatory stimulus can act as anti-inflammatory, and consequently, can be potential therapeutic agents. In the last decades, many identified drug leads and new drugs launched in the market have been derived from natural products. Traditional medicinal herbs have long been used as remedies against inflammatory-related diseases. Moreover, phenolic compounds are secondary metabolites of plants with well known healthy effects, namely antioxidant, cardio- and neuro-protection, anti-cancer and anti-inflammatory. Thus, the study of phenolic content in medicinal plants is extremely relevant in the search of new anti-inflammatory drugs.

This work aimed to identify in the medicinal plant *Cymbopogon citratus* (DC.) Stapf (Cy) phenolic compounds with an anti-inflammatory potential and to investigate their mechanisms of action, through *in vitro* bio-guided assays.

A lipid- and essential oil-free infusion of Cy leaves was prepared and its anti-inflammatory properties were evaluated in LPS-stimulated human and murine macrophages. The production of several pro-inflammatory mediators, namely nitric oxide (NO) and prostaglandin (PG) E_2 in RAW 264.7 murine macrophages, as well as tumor necrosis factor (TNF)-α and chemokine (C-C motif) ligand (CCL) 5 in human macrophages were inhibited by Cy extract (1.115 mg/mL). Its anti-inflammatory activity was correlated with the inhibition of p38 mitogen-activated protein kinase (MAPK) and c-jun NH_2-terminal kinase (JNK) 1/2 signaling pathways. Moreover, it was verified, for the first time, that Cy extract inhibited the chymotrypsin-like activity of proteasome, with the

consequent inhibition of the nuclear factor (NF)-kB, a transcription factor that regulates the expression of numerous inflammatory mediators.

Cy extract was fractionated by column chromatography and polyphenol-rich fractions, namely phenolic acids (PAF yield of 23.8%, w/w, of Cy extract), flavonoids (FF; yield of 4.4%, w/w, of Cy extract) and tannins (TF; yield of 3.5%, w/w, of Cy extract) were obtained. Data revealed that PAF and TF were partially responsible by Cy anti-inflammatory properties through the inhibition of NF-kB activation, NO production, the expression of inducible nitric oxide synthase (iNOS) and TNF-α. In turn, flavonoids decreased TNF-α and iNOS expression but not NO production. Among the three fractions, PAF strongly contributed to the inhibition of proteasome activity triggered by Cy extract. Chemical characterization by HPLC/PDA/ESI-MS^n identified chlorogenic acid (CGA) as the main phenolic acid of the Cy extract, which was partially responsible for the inhibitory effect on the proteasome activity. Therefore, Cy polyphenols, in particular CGA, were highlighted as bioactive compounds.

Flavonoids of Cy were further characterized by proton nuclear magnetic resonance spectroscopy, *O*-, *C*- and *O*,*C*-glycosides of luteolin being isolated and identified, namely luteolin 7-*O*-*β*-glucopyranoside and luteolin 6-*C*-*β*-glucopyranoside (isoorientin), consistent with previous studies, as well as luteolin 2"-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B), identified for the first time in Cy. The cytotoxicity and the anti-inflammatory properties of luteolin and its glycosides were evaluated in LPS-stimulated mouse macrophages. Luteolin glycosides demonstrated less cytotoxicity than luteolin aglycone, which could be an advantage from a pharmacological point of view due to the high toxicity of the current available ant-inflammatory drugs. To evaluate the anti-inflammatory potential of luteolin glycosides, the production of NO as well the expression of iNOS, interleukin (IL)-1β, IL-6 and TNF-α were analyzed. Data revealed that glycosylation decreased the luteolin anti-inflammatory activity, with a higher reduction for *C*-glycosylation than *O*-glycosylation. Taking into account the bioavailability and metabolism of luteolin glycosides, their *in vivo* administration should be considered.

In conclusion, this work demonstrated the anti-inflammatory activity of *Cymbopogon citratus* (DC.) Stapf, using an *in vitro* bio-guided assay, which supports the traditional use of Cy leaves infusion. Furthermore, the molecular mechanisms responsible for the anti-inflammatory properties were elucidated and polyphenols, namely chlorogenic acid and luteolin glycosides, were pointed out as bioactive compounds, encouraging *in vivo* validation studies with the ultimate goal of developing a new anti-inflammatory drug.

Resumo

A inflamação é um processo imunológico complexo envolvido na proteção contra a infeção ou dano tecidular e na manutenção da homeostasia e que, quando desregulado predispõe o hospedeiro para o desenvolvimento de inflamação crónica associada a várias patologias, tais como Alzheimer, diabetes, doenças cardiovasculares, aterosclerose e cancro. No entanto, os fármacos anti-inflamatórios atualmente disponíveis não são totalmente seguros e eficazes, pelo que o desenvolvimento de novos fármacos anti-inflamatórios assume grande relevância. A resposta inflamatória é composta por indutores, tal como o lipopolissacarídeo (LPS), sensores que são expressos nas células inflamatórias, vias de sinalização intracelular que induzem a expressão de genes inflamatórios e moléculas efetoras. Os macrófagos são células inflamatórias que se destacam por proporcionarem uma defesa imediata contra agentes estranhos ao organismo e promoverem uma resposta imune adaptativa através da produção de mediadores inflamatórios, cuja expressão é controlada por vias de sinalização intracelular. Assim, compostos que têm como alvo as vias de sinalização e a produção de mediadores inflamatórios em macrófagos ativados, possuem potencial anti-inflamatório e, consequentemente, valor terapêutico. Nas últimas décadas, a maior parte dos farmacóforos identificados e dos novos medicamentos derivaram de produtos naturais. Várias plantas usadas em medicina tradicional são utilizadas no tratamento de patologias associadas à inflamação. Além disso, os compostos fenólicos são metabolitos secundários das plantas que possuem vários efeitos terapêuticos, tais como, antioxidantes, cardio- e neuro-protetores, anticancerígenos e anti-inflamatórios. Assim, o estudo de compostos fenólicos presentes em plantas medicinais é extremamente relevante na pesquisa de novos fármacos anti-inflamatórios.

O objetivo deste trabalho foi identificar compostos fenólicos de planta medicinal *Cymbopogon citratus* (DC.) Stapf (Cy), com potencial anti-inflamatório e dissecar os seus mecanismos de ação, utilizando ensaios *in vitro* bio-guiados.

Preparou-se uma infusão a partir das folhas de Cy, sem óleos essenciais e lípidos, e as suas propriedades anti-inflamatórias foram testadas em macrófagos estimulados com lipopolissacarídeo (LPS). A produção de mediadores inflamatórios, tais como monóxido de azoto (NO) e prostaglandina (PG) E_2, em macrófagos de ratinho (RAW 264.7), bem como o fator de necrose tumoral (TNF)-α e a quimiocina CCL 5, em macrófagos humanos, foram inibidos pelo extrato de Cy (1,115 mg/mL), estando esta atividade relacionada com a inibição da ativação da p38 MAPK e da JNK 1/2. Além disso, verificou-se, pela primeira

vez, que este extrato inibe a atividade quimiotripsina do proteassoma, com consequente inibição do NF-kB, um fator de transcrição que regula a expressão de mediadores inflamatórios.

O extrato de Cy foi fracionado por cromatografia em coluna obtendo-se frações ricas em polifenóis, nomeadamente os ácidos fenólicos (PAF), os flavonóides (FF) e os taninos (TF). Os resultados obtidos demonstram que PAF e TF são parcialmente responsáveis pelas propriedades anti-inflamatórias do Cy por inibirem a produção de NO, expressão da sintetase do monóxido de azoto (iNOS) e do TNF-α, bem como o NF-kB. Os flavonóides diminuem a expressão de TNF-α e iNOS mas não a produção de NO. Das três frações, PAF contribuiu consideravelmente para a inibição da atividade do proteassoma verificada no extrato do Cy. A caracterização química, realizada por HPLC/PDA/ESI-MS^n, permitiu identificar o ácido clorogénico (CGA) como o principal ácido fenólico do extrato, sendo parcialmente responsável pela inibição da atividade do proteassoma. Desta forma, os polifenóis de Cy, em particular o CGA, foram identificados como compostos bioativos.

Os flavonóides do Cy foram caracterizados por espectroscopia de ressonância magnética nuclear de protão, sendo identificados e isolados *O*-, *C*- e *O*,*C*-glicósidos de luteolina, nomeadamente a luteolina-6-*C*-*β*-glucopiranósido (isoorientin), a luteolina-7-*O*-*β*-glucopiranósido e a luteolina 2"-*O*-ramnosil-*C*-(6-desoxi-ribo-hexos-3-ulosil) (cassiaoccidentalina B), sendo o último identificado pela primeira vez no Cy. A citotoxicidade e as propriedades anti-inflamatórias da luteolina e dos seus glicósidos foram avaliadas em macrófagos RAW 264.7 estimulados com LPS. Os glicósidos de luteolina demonstraram menor citotoxicidade do que a própria luteolina, o que pode constituir uma vantagem do ponto de vista farmacológico dada a elevada toxicidade dos anti-inflamatórios atualmente disponíveis no mercado. Para avaliar o potencial anti-inflamatório dos glicósidos de luteolina, foi analisa a produção de NO, assim como a expressão da iNOS, da interleucina (IL)-1β, IL-6 e do TNF-α. Os resultados demonstraram que a glicosilação diminui a atividade anti-inflamatória da luteolina, sendo esta redução maior para a *C*-glicosilação comparativamente com a *O*-glicosilação. No entanto, tendo em conta a biodisponibilidade e metabolismo dos glicósidos de luteolina, a sua administração *in vivo* deverá ser considerada e explorada.

Em conclusão, este trabalho demonstra as propriedades anti-inflamatórias do *Cymbopogon citratus* (DC.) Stapf, utilizando um ensaio *in vitro* bio-guiado, o que suporta o uso desta planta em medicina tradicional. Além disso, foram elucidados os mecanismos moleculares responsáveis pelas propriedades anti-inflamatórias do Cy, tendo sido

igualmente identificadas diversas moléculas bioativas, designadamente o ácido clorogénico e os glicósidos de luteolina. Estes resultados deverão ser validados *in vivo* tendo por objetivo final o desenvolvimento de um novo fármaco anti-inflamatório.

Abbreviations

1D	Unidimensional
^{1}H	Proton
2D	Two-dimensional
4CL	4-coumaric acid:CoA ligase
δ	Chemical shift
AA	Arachidonic acid
AChE	Acetylcholinesterase
AIDS	Acquired immunodeficiency syndrome
Akt	RAC-alpha serine/threonine-protein kinase
AP-1	Activator protein-1
ARE	Antioxidant response element
ARG	Arginase
ATF	Activating transcription factor
BA2H	Benzoic acid 2-hydroxylase
BChE	Butyrylcholinesterase
br	Broad singlet
CAPE	Caffeic acid phenethyl ester
CasB	Cassiaoccidentalin B
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CD_3OD	Deuterated methanol
CGA	Chlorogenic acid
CHI	Chalcone isomerase
CHS	Chalcone synthase
CLP	Common lymphoid precursors
CMP	Common myeloid precursor
COSY	Correlation spectroscopy
COX	Cyclooxygenase
cPLA2	Cytosolic phospholipase A2
CXCL	Chemokine (C-X-C motif) ligand
Cy	*Cymbopogon citratus* (DC.) Stapf
d	Doublet
dd	Double doublet
DFR	Dihydroflavonol 4-reductase

EC	Effective concentration
EGCG	Epigallocatechin gallate
EIA	Enzyme immunoassay
ELAM	Endothelial-leukocyte adhesion molecule
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
F3H	Flavanone 3-hydroxylase
FF	Flavonoid-rich fraction
FLS	Flavone synthase
FSDC	Fetal skin-derived dendritic cell line
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
gCOSY	Gradient correlation spectroscopy
GLC	Gas liquid chromatography
GMP	Granulocyte/macrophage progenitor
GM-CSF	Granulocyte-macrophage stimulating factor
GST	Glutathione S-transferase
HETE	Hydroxyeicosatetraenoic acid
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HpETE	Hydroperoxyeicosatetraenoic acid
HO	Heme oxygenase
HPLC	High-performance liquid chromatography
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HSC	Hematopoietic stems cells
HUVEC	Human umbilical vein endothelial cells
Hz	Frequency in cycles per second
IC	Inhibitory concentration
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-γ
IkB	Inhibitor of kB
IKK	IkB kinase
IL	Interleukin
IRAK	IL-1 receptor-associated kinase

IRF-3	Interferon regulatory factor-3
iNOS	Inducible nitric oxide synthase
iPLA2	Calcium-independent phospholipase A2
IRF	Interferon regulatory factor
IsoO	Isoorientin
J	Coupling constant
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Keap	Kelch-like ECH-associated protein
KLF	Krüppel-like factor
L7OG	Luteolin 7-*O*-*β*-glucopyranoside
LAR	Leucoanthocyanidin reductase
LC	Lethal concentration
LBP	LPS binding protein
LD	Lethal dose
LDOX	Leucoanthocyanidin oxidase
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
m	multiplet
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
M-CSF	Macrophage colony stimulating factor
MD-2	Myeloid differentiation factor-2
MDP	Macrophages/DC progenitor
MEP	Megakaryocyte/erythrocyte progenitor
MHC	Major histocompatibility complex
MMP	Metaloproteinase
MRC	Mannose receptor C
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
NF-kB	Nuclear factor-kB
NK	Natural killer
NLRs	Nucleotide-binding oligomerization domain receptors

NMR	Nuclear magnetic resonance
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NQO	NAD(P)H:quinone oxidoreductase
Nrf2	Nuclear factor erythroid 2-related factor 2
NSAID	Non-steroidal anti-inflammatory drug
PAL	Phenylalanine ammonia lyase
PCA	Protocatechuic acid
PDA	Photodiode array
PDGF	Platelet-derived growth factor
PDL	Programmed death ligand
PFs	Polyphenol-rich fractions
PG	Prostaglandin
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PLA2	Phospholipase A2
PPAR	Peroxisome proliferator-activated receptor
ppm	Parts per million
PPRE	Peroxisome proliferator response element
PRR	Pattern recognition receptors
PTKs	Protein tyrosine kinase
RELM	Resistin-like molecule
RIP-1	Receptor-interacting protein 1
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
RT-PCR	Real time-polymerase chain reaction
ROS	Reactive oxygen species
RXR	Retinoid X receptor
s	Singlet
SARM	Sterile alpha- and armadillo-motif-containing protein
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGLT	Sodium-dependent glucose cotransporter
SNAP	*S*-nitroso-*N*-acetylpenicillamine
sPLA2	Secretory phospholipase A2

SRA	Scavenger receptor class A
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
t	Triplet
TAK-1	Transforming growth factor β–activated kinase-1
TANK	TRAF family member-associated NF-kB activator
TBK-1	TANK binding kinase-1
TF	Tannin-rich fraction
TGF-β	Transforming growth factor-β
Th	T-helper cell
TIMP	Tissue inhibitor of metalloproteinases
TIR	Toll-interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TIS	Temporary immersion system
TLC	Thin layer chromatography
TLR	Tool-like receptor
TNF	Tumour necrosis factor
TOCSY	Total correlation spectroscopy
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRE	Tetradecanoylphorbol-13-acetate responsive element
TRIF	TIR domain-containing adaptor inducing IFN-β
TRX	Thioredoxin
TX	Tromboxan
UGT	UDP glucuronosyltransferase
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule

CHAPTER I

INTRODUCTION

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A. Inflammation

1. Overview of the inflammatory process

Inflammation is a complex immunological process by which the body fights infection or injury from bacteria, viruses, other pathogens, and danger signals. During acute inflammation, serum proteins and leukocytes move from the blood to the extra-vascular tissue leading to clinical features of inflammation, known in Latin as *rubor* (redness), *calor* (warmth), *tumor* (swelling) and *dolor* (pain) (Yoon and Baek, 2005). There are two types of inflammation, acute and chronic inflammation. Acute inflammation is a process mediated through the activation of the immune system cells that persists only for a short period until reaching resolution, usually being beneficial for the host. If the inflammation lasts for a longer period of time the inflammation can become chronic. The sustained inflammatory/oxidative environment leads to a vicious circle, which can damage healthy neighbouring epithelial and stromal cells and over a long period of time may lead to chronic illnesses, including Alzheimer, diabetes, cardiovascular diseases, atherosclerosis and cancer (Schmidt and Duncan, 2003; Porta *et al.*, 2009; Whitney *et al.*, 2009; Hunter and Doddi, 2010).

The inflammatory response consists of: 1) inducers derived for infection or tissue damage; 2) sensors expressed in inflammatory cells such as tissue-resident macrophages, dendritic, and mast cells; 3) signaling transduction pathways that induce the expression of genes involved in the inflammatory response; and 4) effectors (Medzhitov, 2010; Tabas and Glass, 2013) (Fig 1.1). Amid the inflammatory cells that senses the environment, macrophages have a key role in inflammation by providing not only an immediate defense against foreign agents but also promoting an adaptative immune response (Murray and Wynn, 2011). A classic inducer of inflammation is the bacterial lipopolysacharide (LPS), a component of the outer membrane of Gram-negative bacteria that is sensed by the innate receptor Toll-like receptor (TLR)-4. The activation of inducers´ receptors, trigger the expression of inflammatory mediators, through finely regulated changes in the activation of transcription factors, such as the nuclear factor (NF)-kB, activator protein (AP)-1, the peroxisome proliferator activated receptors (PPARs) and the nuclear factor erythroid 2-related factor 2 (Nrf2), whose DNA-binding capacity is modified by signal transduction pathways, including mitogen activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (Akt) and the ubiquitin-proteasome system. The produced inflammatory mediators, namely nitric oxide (NO);

adhesion molecules: intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin; lipid-derived eicosanoids: prostaglandin (PG) E_2, PGI_2, leukotriene (LT) B_4, LTC_4; cytokines: tumor necrosis factor (TNF)-α, interleukin (IL) 1β, IL6 and IL10; and chemokines: chemokine (C-X-C motif) ligand (CXCL) 8, chemokine (C-C motif) ligand 2 (CCL2), CCL3, among others, are effectors involved in the coordination of acute inflammatory vascular changes and cell recruitment (Santangelo *et al.*, 2007),.

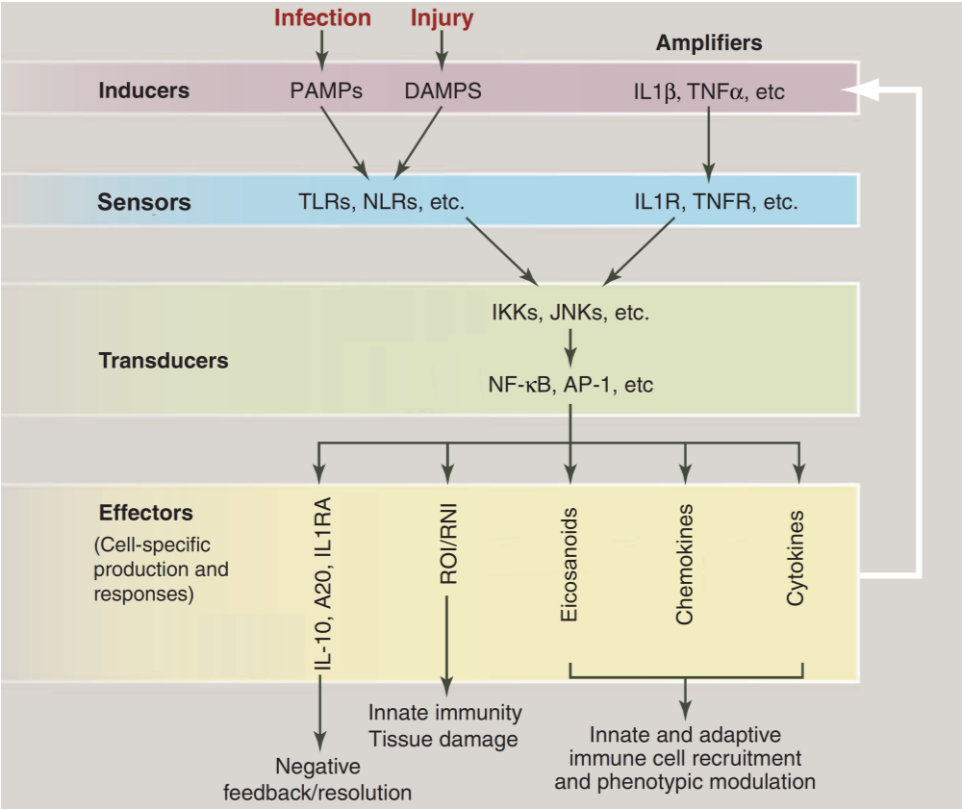


Figure 1.1. Inflammatory pathway components: inducers, sensors, transducers and mediators. Inflammation is initiated by pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs), which are recognized by pattern recognition receptors, such as toll-like receptors (TLR) and NOD-like receptors (NLRs). After, inducer´s binding, these receptors engage signaling transduction pathways that activate transcription factors, like nuclear factor (NF)-kB and activator protein (AP)-1, leading to the expression of: a) anti-inflammatory/resolution mediators, such as interleukin (IL)-10; b) genes that exert antimicrobial activities through production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI), like nitric oxide; c) inflammatory mediators, including cytokines, chemokines and eicosanoids, that initiate inflammatory response and recruit additional immune cells, thereby setting into motion both innate and daptative immune responses. The expression of inflammatory cytokines provides a feed-forward loop for amplification of the initial response. IKK, inhibitor of kB kinase; IL1R, interleukin 1 receptor; JNK, c-Jun N-terminal kinase; TNF, tumour necrosis factor; TNFR, tumour necrosis factor receptor. Adapted from Medzhitov, 2010; Tabas and Glass, 2013.

2. Inflammatory cells: macrophages

2.1. Classification and origin

Macrophages are derived from bone-marrow hematopoietic stems cells (HSC) precursors that develop into monocytes and then macrophages (Verschoor *et al.*, 2012). Traditionally, in bone marrow, HSC give rise to two main classes of progenitors which are progressively committed with a particular cell lineage (Buza-Vidas 2007): common lymphoid precursor (CLP), which generates T lymphocytes, B lymphocytes, and natural killer cells; and common myeloid precursor (CMP), which generates either megakaryocyte/erythrocyte progenitor (MEP), or granulocyte/macrophage progenitor (GMP), with monocytes arising from the latter (Fig. 1.2). In the differentiation process, monocyte commitment is induced primarily by the presence of growth factors: macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage stimulating factor (GM-CSF). Upon recruitment to a specific tissue, monocytes replenish tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum (Mosser and Edwards, 2008). This paradigm of myeloid development is well supported; however, there is evidence of additional or alternative roadmaps that remain poorly understood and further investigation is needed (Giebel and Punzel, 2008; Verschoor *et al.*, 2012).

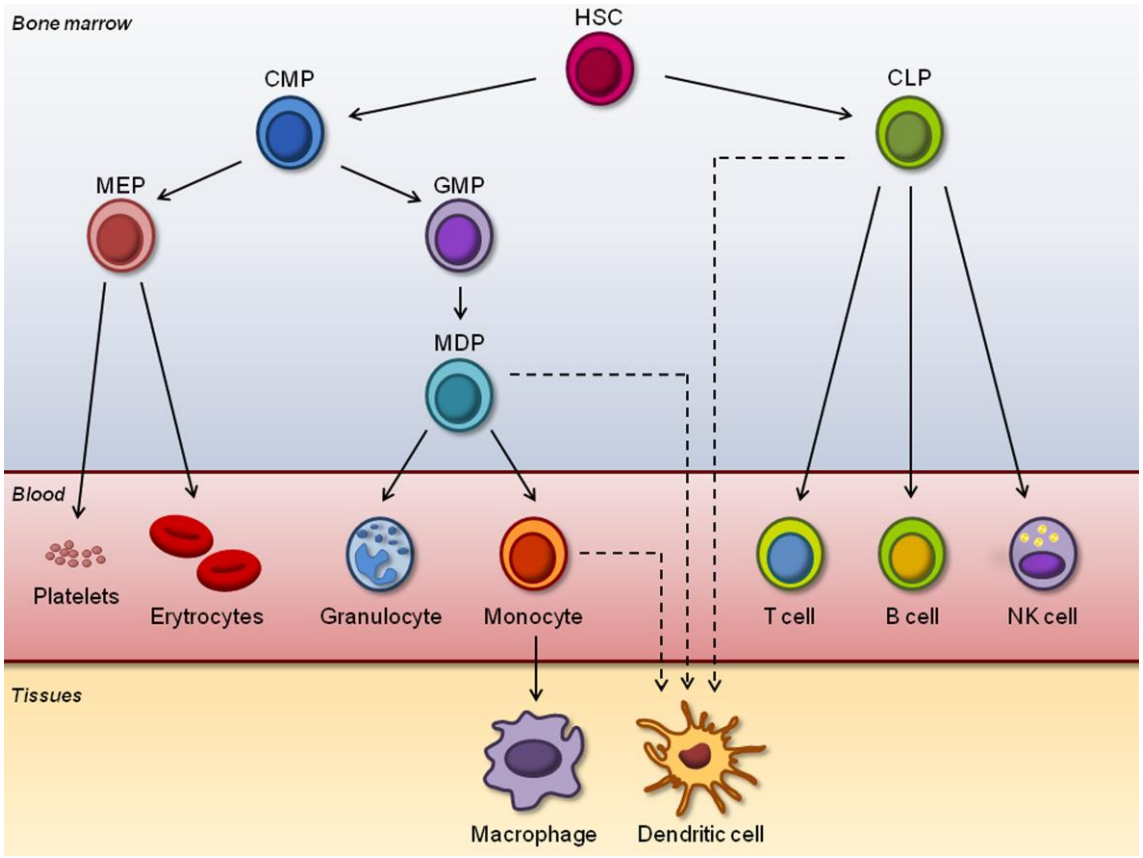


Figure 1.2. Origin and differentiation of macrophages. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage precursor; HSC, hematopoietic stems cells; MDP, macrophage/DC progenitor; MEP, megakaryocyte/erythrocyte progenitor. Adapted from Larsson and Karlsson, 2005; Geissmann *et al.*, 2010; Ransohoff and Cardona, 2010.

2.2. Immunobiology

2.2.1. Polarization and plasticity

Macrophages, located throughout the body, are considered professional phagocytes because they sense the local environment by phagocytosis, being essential for the maintenance and defense of host tissues. They are remarkably plastic cells that can rapidly change their phenotype, with consequent activation, in response to environmental clues. In a simplistic view, these antigen-presenting cells of the innate immune system can undergo functional polarization into classical activated macrophages, or M1, and alternative macrophages, or M2, as well as regulatory cells (Fig. 1.3).

The M1 phenotype, which activating stimuli are TLR ligands and interferon gamma (IFNγ), is characterized by enhanced antigen-presenting capacity, the secretion of high amounts of reactive oxygen species (ROS), metaloproteinases (MMPs), nitric oxide (NO), chemokines and pro-inflammatory cytokines, as well as the induction of strong IL12-mediated T-helper 1 (Th1) responses. M1 macrophages have strong microbicidal and

tumoricidal activities, but at the same time their defense mechanisms can induce collateral damage to the host (Cassetta *et al.*, 2011; Verschoor *et al.*, 2012). In contrast, M2 macrophages induce T helper 2 (Th2) responses and have the ability to limit inflammation, through endocytic clearance and trophic factor synthesis, and to promote tissue remodeling, and hence they are considered wound-healing macrophages. The M2 phenotype is promoted by exposure to IL4 and IL13 cytokines, produced by $CD4^+$ T-cells. Moreover, M2 macrophages are involved in the response to parasites and fungi, being characterized by efficient phagocytic activity and the expression of mannose receptors, as well as high amounts of cytosolic arginase and extracellular matrix related proteins (Cassetta *et al.*, 2011; Verschoor *et al.*, 2012). Therefore, in general, M1 activation is implicated in initiating and sustaining inflammation, while M2 activation is associated with resolution or smoldering inflammation.

The signaling pathways that determine M1 or M2 polarization have been outlined. A high crosstalk between the M1/M2 polarizing pathways were verified, being the members of PPAR, Krüppel-like factor (KLF), interferon regulatory factor (IRF), signal transducer and activator of transcription (STAT), NF-kB, and hypoxia-inducible factor (HIF) families determinants to the final phenotype. It has been demonstrated that a predominance of NF-kB and STAT1 activation promotes M1 phenotype, while a prevalence of STAT3 and STAT6 activation results in M2 phenotype. Furthermore, epigenetic modifications, with involvement of histone methylation and acetylation, are also deciding in macrophage polarization (Sica and Mantovani, 2012). In addition, several *in vitro* and *in vivo* studies suggest that macrophages can switch their phenotype over time. Although this mechanism is not fully understood, deregulation of M1-M2 switch is associated with pathological conditions such as autoimmune diseases, chronic infection, allergy, cancer, obesity and atherosclerosis (Mosser and Edwards, 2008; Murray and Wynn, 2011; Hoeksema *et al.*, 2012; Sica and Mantovani, 2012).

In addition to M1 and M2, regulatory macrophages are an important subset that plays a pivotal role in limiting inflammation during innate and adaptative immune responses. This cell type is induced by immune complexes and TLR ligands, or IL10 and glucocorticoids and can produce high levels of IL10, like M2, but not extracellular matrix proteins. Additionally, regulatory macrophages concurrently express NO synthase and arginase, reinforcing the distinct activation phenotype (Cassetta *et al.*, 2011).

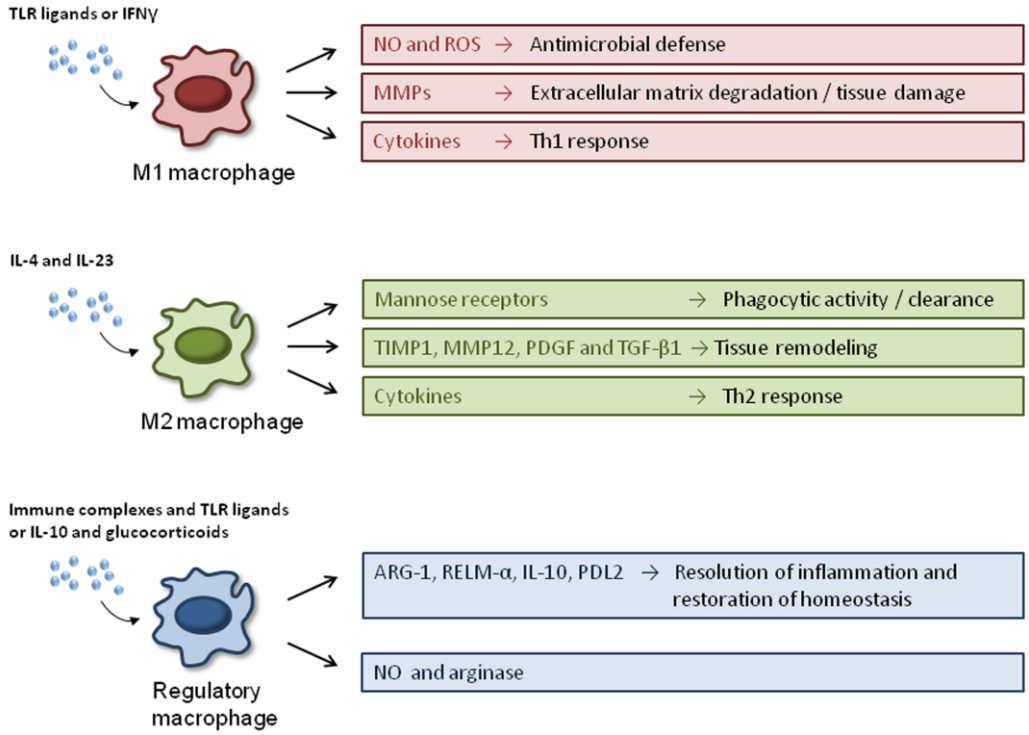


Figure 1.3. Macrophage polarization. ARG, arginase; IL, interleukin; MMP, metalloproteinase; NO, nitric oxide; PDGF, platelet-derived growth factor; PDL, programmed death ligand; RELM, resistin-like molecule; ROS, reactive oxygen species; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases. Adapted from Mosser and Edwards, 2008; Murray and Wynn, 2011.

Classification into M1, M2 or regulatory macrophages is important to distinguish the diferent populations, but macrophages are high plastic cells and this classification does not fully represents the complexity of transitional states of macrophage activation, which is often fine tuned accordingly to the microenvironment. Accordingly, more recently, Mosser and Edwards (2008) proposed a more flexible classification in which macrophages are considered as part of a continuum having a range of overlapping functions between classically activated (M1), wound-healing (M2), and regulatory macrophages.

2.2.2. Functional features

The primary function of macrophages is to maintain homeostasis, which include host defense against foreign invaders, clearance of necrotic and apoptotic debris, and tissue remodeling following injury. They are able to perform these functions due to their basic innate abilities of sensing, phagocytosis and repair, chemotaxis and adaptative stimulation (Verschoor *et al.*, 2012).

Macrophages sense the local environment through intracellular and cell-surface pattern recognition receptors (PRRs) that recognize molecular patterns common across a number of species. Generally, ligands of PRRs could be divided into: exogenous, namely pathogen molecular patterns, and endogenous, such as necrotic/apoptotic cell debris and modified host proteins and lipids. Among the PRRs, are the TLR family that promotes pro-inflammatory activities and dectin-1 as well as mannose receptor C (MRC) 1 that promote anti-inflammatory responses (Takeuchi and Akira, 2010). Additionally, macrophages express cell-surface Fc receptors, which bind circulating antibodies attached to foreign antigens, leading to phagocytosis, and scavenger receptors, such as scavenger receptor class A (SRA) and CD36, which recognize both exogenous (e.g. bacterial cell wall components) and endogenous ligands (e.g. oxidized low-density lipoproteins).

Upon sensing, macrophages stimulate the expansion of activated T cells and secrete chemokines that recruit the appropriate effector cells. The secreted chemokines differ accordingly to the polarization phenotype. M1 macrophages secrete CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL11, which are chemoattractants for monocyte/macrophages, Th1 and natural killer (NK) cells, as well as CXCL8 that recruits neutrophils. In turn, M2 macrophages secrete CCL17 and CCL22, which attract Th2 and NK cells, and CCL24, which attracts eosinophils and basophils.

After recognition of its ligands, some receptors promote the process of phagocytosis by which macrophages engulf the material, sequestering it within a phagosomal compartment that merges with lysosomes, which contain hidrolitic enzymes and highly reactive and toxic molecules (e.g. ROS, H_2O_2 and oxide anions), allowing to the destruction of the phagosomal content. Besides clearance, this process also generates antigenic sequences for presentation to T lymphocytes through major histocompatibility complex (MHC) II receptors. Additional signaling, through IL12 or IL4, leads to the expansion of antigen specific T lymphocytes, thus promoting the adaptative immune response.

3. Inflammatory inducers and their receptors: LPS-TLR4 signaling pathway

TLRs recognize components of microorganisms, such as LPS, leading to the activation of immune system (Aderem and Ulevitch, 2000; Kawai and Akira, 2010). LPS is a structural component of the outer membrane of Gram-negative bacteria that stimulates immune cells via interaction with LPS binding protein (LBP), cluster of differentiation (CD) 14, myeloid differentiation factor (MD)-2 and TLR4. LBP directly binds to LPS and facilitates the association of LPS to CD14, which helps the transfer of LPS to TLR4/MD-2 receptor complex. Upon LPS recognition, TLR4 undergoes oligomerization and recruits downstream adaptors with the toll-interleukin-1 receptor (TIR) domains. These adaptors are: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile alpha- and armadillo-motif-containing protein (SARM) (Lu *et al.*, 2008). Considering the adaptors involved, TLR4 signaling could be divided in MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway, used by all the known TLRs with the exception of TLR2, induces the expression of pro-inflammatory cytokines, while the MyD88-independent pathway, used by TLR3 and TLR4, modulates type I interferons and interferon-inducible genes (Pålsson-McDermott and O'Neill, 2004; Lu *et al.*, 2008) (Fig. 1.4).

In the MyD88-dependent pathway, the adaptor MyD88 recruits and activates IL-1 receptor-associated kinase (IRAK)-4 that, in turn, activates IRAK-1. Once activated, IRAK-1 interacts with the TNF receptor associated factor (TRAF)-6, which activates transforming growth factor β–activated kinase (TAK)-1. Subsequently, TAK-1 activates IkB kinase (IKK) and MAPK pathways, which lead to the activation of NF-kB and AP-1 transcription factors that ultimately control the expression of pro-inflammatory cytokines (Pålsson-McDermott and O'Neill, 2004; Lu *et al.*, 2008).

In the MyD88-independent signaling, the adaptor protein TRIF interacts with receptor-interacting protein 1 (RIP-1), an important component of TNF-α-mediated NF-kB activation, or with TRAF-3. TRAF-3 associates with TRAF family member-associated NF-kB activator (TANK), TANK binding kinase (TBK)-1 and IKKε to activate interferon regulatory factor (IRF)-3. The activation of IRF3 and NF-kB ultimately induces type I IFNs and IFN-inducible genes, which are important for anti-viral and anti-bacterial responses (Pålsson-McDermott and O'Neill, 2004; Lu *et al.*, 2008).

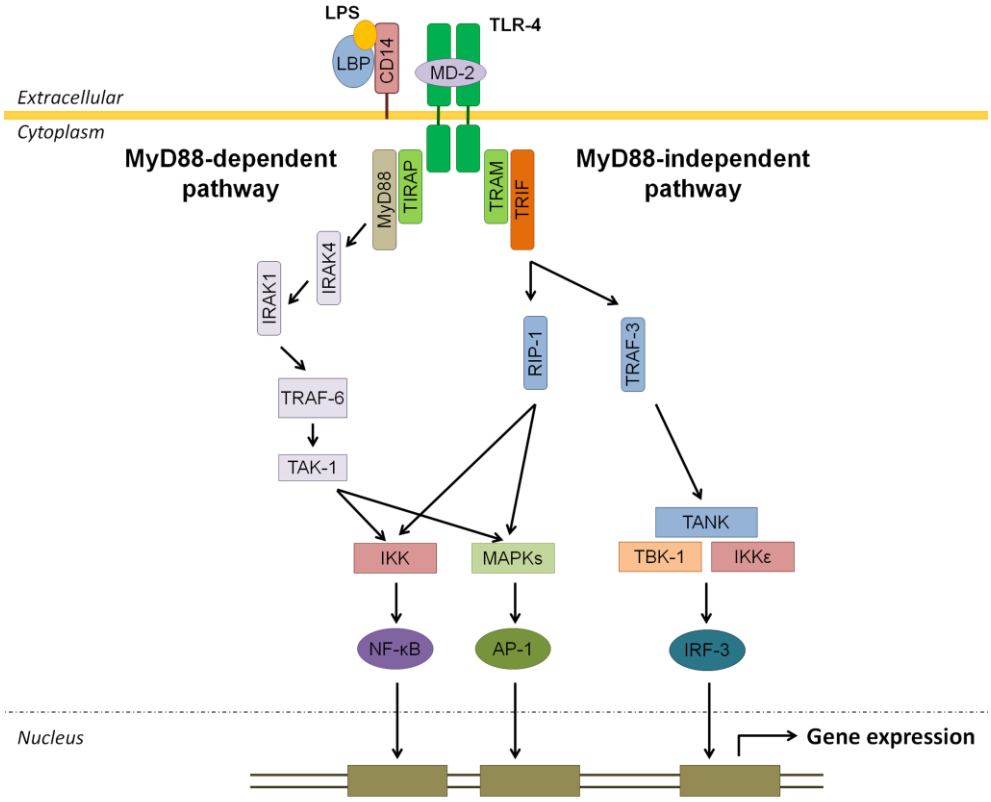


Figure 1.4. Schematic representation of LPS-TLR-4 signaling pathway. AP-1, activator protein-1; CD14, cluster of differentiation 14; IKK, IkB kinase; IRAK, IL-1 receptor-associated kinase; IRF-3, interferon regulatory factor-3; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NF-kB, nuclear factor-kB; RIP-1, receptor-interacting protein 1; TAK-1, transforming growth factor β–activated kinase-1; TANK, TRAF family member-associated NF-kB activator; TBK-1, TANK binding kinase-1; TIRAP, TIR domain-containing adaptor protein; TLR-4, toll-like receptor-4; TRAF, TNF receptor associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor inducing IFN-β. Adapted from Pålsson-McDermott and O'Neill, 2004; Lu *et al.*, 2008.

4. Intracellular signaling pathways involved in inflammation

The inflammatory response is a well-coordinated serie of events tightly controlled by the balance of particular intracellular signaling pathways (Newton and Dixit, 2012). Among these pathways are the MAPKs and several transcription factors (Fig. 1.5).

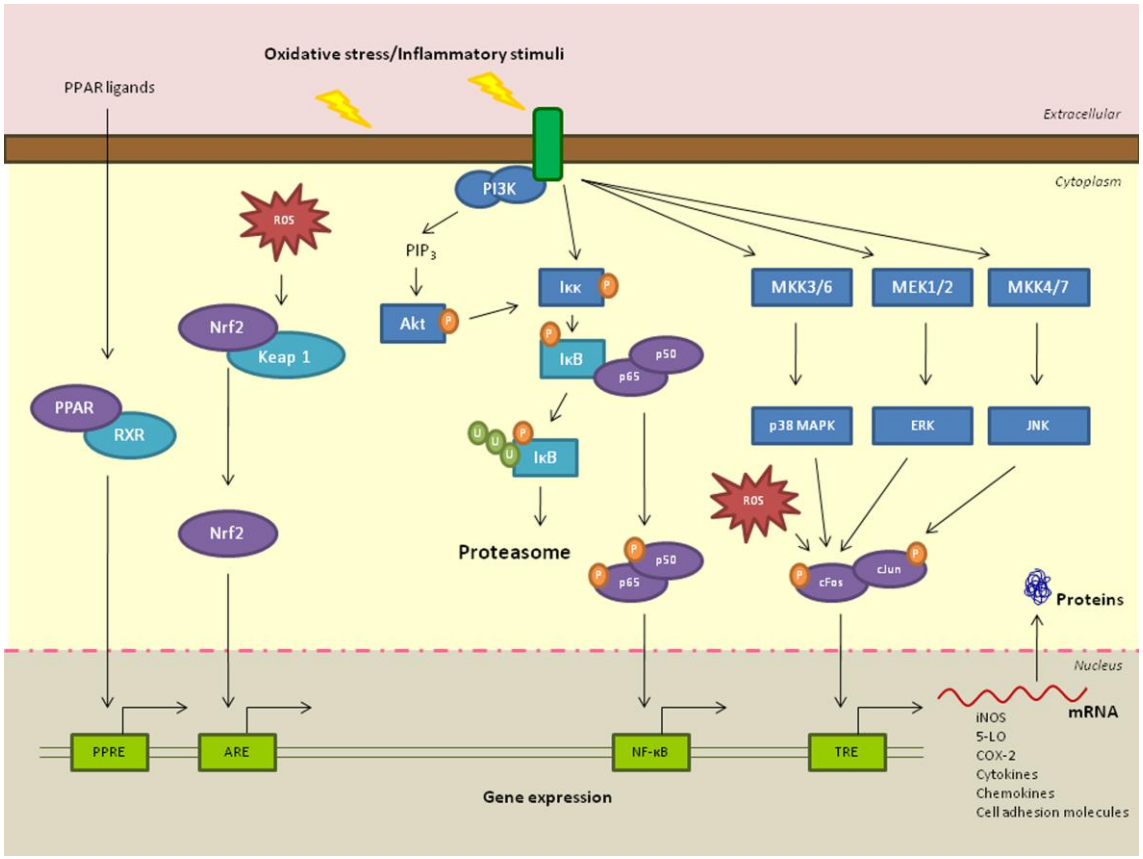


Figure 1.5. Inflammatory intracellular signalling pathways and mediators. Akt, RAC-alpha serine/threonine-protein kinase; ARE, antioxidant response element; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; IkB, inhibitor of kB; IKK, IkB kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; Keap1, kelch-like ECH-associated protein 1; LO, lipoxygenase; MAPK, mitogen-activated protein kinase; MKK and MEK, MAPK kinase kinase; NF-kB, nuclear factor-kB; Nrf2, nuclear factor erythroid 2-related factor 2; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; ROS, reactive oxygen species; RXR, retinoid X receptor; TRE, tetradecanoylphorbol-13-acetate responsive element.

4.1. Transcription factors

4.1.1. Nuclear factor (NF)-kB

The NF-kB family comprises multiple proteins with an amino-terminal REL homology domain, namely p50, p52, p65, c-REL and RELB (Vallabhapurapu and Karin, 2009). These proteins can homodimerize as well as form heterodimers with each other, being p50/p65 heterodimer the most prevalent activated form. The NF-kB dimers are kept in the cytoplasm through interaction with inhibitor of kB (IkB) proteins family, such as IkBα, IkBβ and IkBε. In the canonical activation of NF-kB pathway, this transcription factor could be activated by inflammatory stimuli like LPS, cytokines and oxidants through several signaling pathways that lead to the phosphorylation of inhibitor of kB (IkB) by the IkB kinase (IKK), which is a marker for ubiquitination and the subsequent degradation by proteasome. IkB degradation unmasks the nuclear localization motif of NF-kB dimers, allowing its rapid translocation to the nucleus and the subsequent transcription of target genes (Vallabhapurapu and Karin, 2009). The NF-kB pathway regulates the expression of numerous proteins related to inflammation, such as the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, and several inflammatory mediators like TNF-α, IL-1β, IL6, among others (Tak and Firestein, 2001; Vallabhapurapu and Karin, 2009). In order to prevent immunopathology and to maintain homeostasis, the activation of NF-kB needs to be properly attenuated and/or terminated, which occurs through several negative regulation mechanisms, such as resynthesis of IkB or p65 ubiquitination and degradation in the nucleous (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009). In fact, NF-kB is highly activated at sites of inflammation in several diseases, like rheumatoid arthritis, atherosclerosis and inflammatory bowel disease (Tak and Firestein, 2001). Therefore, NF-kB is considered the master regulator of innate immunity and inflammatory responses and an important target in the development of new anti-inflammatory drugs (O'Neill, 2006).

4.1.2. Activator protein (AP)-1

The redox-sensitive transcription factor AP-1 is a heterodimeric protein complex composed by members of basic region leucine zipper protein super-family, namely Jun (vJun, cJun, Jun B, Jun D) and Fos [vFos, cFos, Fos B, Fra-1/2 (Fos-related antigen)], and the activating transcription factor (ATF) family (ATF2, ATF3 and B-ATF). Additionally, several proteins of the maf family (v-maf, c-maf and Nrl) can heterodimerize with c-Jun or

c-Fos, whereas other Maf related proteins (MafB, MafF, MafG and MafK) heterodimerize with c-Fos but not with c-Jun (Shaulian and Karin, 2001). AP-1 heterodimer binds to its palindromic DNA sequence (TRE for tetradecanoylphorbol-13-acetate responsive element), regulating the expression of genes involved in apoptosis, cellular response to stress and inflammation (Schonthaler *et al.*, 2011). AP-1 activity is regulated by MAPKs, in particular by JNK through phosphorylation of cJun (Karin, 1995; Erdèlyi *et al.*, 2005). In addition, a positive synergy between AP-1 and NF-kB has been reported (Stein *et al.*, 1993).

4.1.3. Peroxisome proliferator-activated receptors (PPARs)

PPARs are present in three isoforms, namely PPARα, PPARδ (also known as PPARβ) and PPARγ, and belong to a large group of related transcription factors referred to as the nuclear receptor superfamily (Blanquart *et al.*, 2003). Nuclear receptors bind small lipophilic ligands that induce their transcriptional activities. Upon ligand binding, PPARγ, the most studied PPAR, forms obligate heterodimers with the nuclear receptor retinoid X receptor α (RXRα) and then, binds to the peroxisome proliferator response elements (PPREs), thus modulating the transcription of target genes. Usually, PPARγ activation leads to stimulation of transcription, but depending on the formed heterodimers, its activation can also lead to the repression of gene expression. PPARγ is involved in the direct regulation of genes responsible for lipid transport and metabolism. However, both PPAR and RXRα also play a central role in the down-regulation of: inflammatory signaling pathways, namely MAPKs and transcription factors (e.g. NF-kB and AP-1) activation; inflammatory chemokines and cytokines (e.g. IL1β and TNF-α) production; and adhesion molecules expression (Becker *et al.*, 2006). In addition, and due to their importance in the modulation of the inflammatory response (Blanquart *et al.*, 2003), PPARs were described as new therapeutical targets in inflammatory bowel disease (Dubuquoy *et al.*, 2006).

4.1.4. Nuclear factor erythroid 2-related factor 2 (Nrf2)

Nrf2 is a redox sensitive transcription factor that belongs to the cap´n´collar subfamily containing the basic leucine zipper region (Kaspar *et al.*, 2009). The activity of Nrf2 is normally repressed by kelch-like ECH-associated protein (Keap)-1, a cytoskeleton-associated protein. Upon stimulation by electrophilic agents or thiol group modifiers, the interaction of Nrf2-Keap1 is disrupted, enabling Nrf2 to translocate to the nucleus and potentiating the expression of antioxidant response element (ARE)-dependent genes, such as NAD(P)H:quinone oxidoreductase (NQO), glutathione S-transferase (GST), UDP

glucuronosyltransferase (UGT), thioredoxin (TRX), heme oxygenase (HO)-1 and ferritin (Kaspar *et al.*, 2009; Tsuji *et al.*, 2000; Zhang *et al.*, 2010). During inflammation, there is an increased release and acummulation of reactive species in the site of damage that activate Nrf2-ARE pathway, and thus, induce endogenous antioxidant defense systems that rescues cells/tissues from inflammatory injuries. Additionally, due to its protective properties against electrophilic and oxidative stress, recent studies have described the role of Nrf2 in inhibiting the production or expression of pro-inflammatory mediators, including cytokines, chemokines, cell adhesion molecules, COX-2 and iNOS (Kim, Cha, *et al.*, 2010). Therefore, activation of Nrf2-ARE pathway is an important strategy for the treatment of inflammation.

4.2. Protein tyrosine kinases (PTKs)

PTKs catalyze the transfer of a phosphate group from ATP to tyrosine residues of proteins, and comprise two general classes of molecules: receptor tyrosine kinases and non-receptor tyrosine kinases (Hubbard and Miller, 2007). Its activation occurs following phosphorylation of its tyrosine residues, which serves as an initial trigger in cellular activation in response to extracellular stimuli, cell-cell interactions, tissue homeostasis, among others stimuli (Hubbard and Miller, 2007; Page *et al.*, 2009).

PTKs are involved in TLRs signaling, and therefore are crucial for cytokine production (Page *et al.*, 2009). Additionally, these kinases are involved in the intracellular response to many cytokines, such as TNF-α, IL6 and IL10; being implicated not only in cytokine production, but also in their function (Page *et al.*, 2009). Thus, modulation of PTKs activity can be a promising therapeutic strategy to treat inflammatory conditions.

4.3. Mitogen-activated protein kinases (MAPKs)

Different pro-inflammatory stimuli, such as microbial products (e.g. LPS) and cytokines (IL1β and TNF-α), bind to TLRs, IL1 receptor family or TNF receptor family present in inflammatory cells, triggering the activation of specific signaling transduction profiles responsible for the production of inflammatory mediators (Newton and Dixit, 2012). The MAPK pathway is one of the major pathways activated by inflammatory stimuli. Three major groups of distinctly regulated MAPK cascade that lead to altered gene expression are known as extracellular signal-regulated kinase (ERK) 1/2, JNK and p38 MAPK (Newton and Dixit, 2012). The ERK1/2 pathway is most commonly linked to the regulation of cell differentiation and survival, whereas JNK and p38 MAPK pathways,

usually referred to as stress-stimulated MAPKs, are activated by various environmental stresses such as osmotic shock, ultraviolet radiation, heat shock, oxidants and pro-inflammatory cytokines and are also known to mediate apoptosis. Each MAPK pathway consists of a module of three kinases: a MAPK kinase kinase (MAPKKK), which phosphorylates and activates a MAPK kinase (MAPKK) that in turn phosphorylates and activates a MAPK. Once activated, MAPKs phosphorylate and activate several transcription factors like NF-kB, AP-1, PPAR and Nrf2, leading to the expression of target genes, including inflammatory mediators (Owuor and Kong, 2002; Kaminska, 2005; Broom *et al.*, 2009). The different MAPKs overlap multiple substrate specificities and phosphorylation of regulatory sites, being the interaction of multiple MAPKs cascades important to integrate the responses and to activate separate sets of genes. Given its crucial role, the inhibition of MAPKs could lead to anti-inflammatory effects by modulating the levels of pro- and anti-inflammatory mediators.

4.4. Phosphatidylinositol 3-kinase (PI3K)/ RAC-alpha serine/threonine-protein kinase (Akt)

During inflammatory responses, the engagement of a wide variety of stimuli, like inflammatory mediators, immunoglobulins, antigens, growth factors, neurotransmitters and hormones, to its corresponding cell surface receptors leads to the activation of the PI3K pathway (Marone *et al.*, 2008). PI3K is a heterodimer formed by the regulatory subunit p85 and the catalytic subunit p110 (Marone *et al.*, 2008). The p85 subunit associates directly with the cell surface receptor, and the subsequent conformational change in the p85-p110 heterodimer confers activation. Once activated the PI3K produces phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), which recruits adaptor and effector proteins, such as Akt. When activated, Akt is responsible for the regulation of target molecules involved in cell growth, proliferation, survival, metabolic homeostasis and cell migration, as well as in inflammatory diseases (Marone *et al.*, 2008; Fougerat *et al.*, 2009). Therefore, PI3K/Akt has been pointed as an attractive target for the treatment of inflammatory-related diseases (Ghigo *et al.*, 2010).

4.5. Ubiquitin-proteasome system

The ubiquitin-proteasome system has a central role in the selective degradation of intracellular proteins (Shen *et al.*, 2006). This pathway is regulated and coordinated by several enzymatic reactions, where polyubiquitination, the covalent link of ubiquitin molecules to the lysine residues of proteins, is a signal to subsequent protein degradation

by the multicatalytic protease, the 26S proteasome. The activity of ubiquitin-proteasome system is a highly regulated event that allows cellular proteins turnover, serving multiple intracellular functions, like degradation of damaged proteins and modulation of regulatory proteins involved in several biological processes, including inflammation, viral shedding, cell cycle, growth and differentiation. The role of proteasome in the activation of the NF-kB transcription factor is a well documented event and regarding the importance of NF-kB in inflammation, targeting proteasome could also be an anti-inflammatory therapeutic strategy (Elliott *et al.*, 2003). Furthermore, the ubiquitin proteasome system could modulate the activation of Nrf2 (Nguyen *et al.*, 2003), which has an important role in inflammation, as described above. Another mechanism by which proteasome is involved in inflammatory process is through modulation of the turnover of several inflammatory mediators, like TNF-α, IL6, IL12 p40 and p35, and enzymes like COX-2 and iNOS, among others (Shen *et al.*, 2006).

5. Inflammatory mediators

5.1. Nitric oxide

Nitric oxide is a cellular mediator synthesized from L-arginine by NOS (Guzik *et al.*, 2003). There have been identified three NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The NO synthesized by NOS could exert many biological functions in the cardiovascular, nervous and immune systems. Both eNOS and nNOS are constitutively expressed and its activation requires an increase in the intracellular calcium concentration, while iNOS expression is a high-output isoform induced by inflammatory stimuli in certain cells such as macrophages, and its activity is independent of changes in intracellular calcium (Geller and Billiar, 1998). The high output of iNOS-derived NO is usually beneficial for the host if produced for a short time; however, its production for a longer period contributes to the pathogenesis of septic shock and inflammatory diseases (Zamora *et al.*, 2000; Guzik *et al.*, 2003). For that reason, the selective inhibition of NO production and iNOS expression and/or its activity is a useful biomarker for an initial screening of anti-inflammatory drugs.

5.2. Eicosanoids

Eicosanoids, like PGs and LTs, are important inflammatory mediators derive from arachidonic acid (AA) (Harizi *et al.*, 2008). The AA biosynthetic cascade has been extensively studied in order to develop new anti-inflammatory compounds. In particular, aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), the most used anti-inflammatory drugs, inhibit COX, a pro-inflammatory enzyme that synthesizes PGs (Rao and Knaus, 2008). The rate-limiting step in eicosanoid biosynthesis is the release of AA by membrane phospholipids mainly through the action of the phospholipase A_2 (PLA_2), or by the combined action of phospholipase C and diacylglycerol lipase. PLA_2 exists in many isoforms and is broadly classified into three large categories: secretory PLA_2 ($sPLA_2$) and cytosolic PLA_2 ($cPLA_2$), which are Ca^{2+}-dependent, and the calcium-independent PLA_2 ($iPLA_2$) (Burke and Dennis, 2009). The $iPLA_2$ is thought to serve a housekeeping role in phospholipids remodeling while $sPLA_2$ and $cPLA_2$ are associated with the inflammatory process, and thus, modulation of their activity is a potential target to control inflammation.

After being generated, AA could be metabolized by the lypoxygenase (LO) pathway into hydroperoxyeicosatetraenoic acids (HpETEs), hydroxyeicosatetraenoic acids (HETEs) and LTs. The 5-LO products 5-HETE, LTA4 and LTB4, are potent chemoattractants, while LTC4, LTD4 and LTE4 are responsible for the asthma syndrome through contraction of the respiratory smooth muscle. The 12-HETE produced by 12-LO aggregates the platelets and induces the inflammatory response. Therefore, 5- and 12-LO products are involved in the inflammatory/allergic disorders. In contrast, 15-LO synthesizes anti-inflammatory 15-HETE (Harizi *et al.*, 2008).

The AA could also be metabolized by COX to PGs and thromboxans (TXs). COX enzyme exists, at least, in two isoforms (COX-1 and COX-2) and one variant (COX-3) (Rao and Knaus, 2008). The constitutive isoform COX-1 is present in almost every cell type and produces cytoprotective and blood aggregatory PGs, while COX-2 is highly expressed in inflammatory-related cells by LPS or inflammatory cytokines, producing high amounts of PGs. As COX-2 is a pivotal enzyme in inflammation and the selective inhibition of COX-2 has been reported to increase the cardiovascular risk, probably by TXs formed via COX- 1, the COX-2 inhibitors are being continuously developed in order to obtain safer anti-inflammatory drugs (Rao and Knaus, 2008; Ritter *et al.*, 2009).

5.3. Cytokines and chemokines

Cytokines are protein inflammatory mediators with important roles in the regulation of vascular changes and inflammatory cells recruitment (Rosa *et al.*, 2012). In particular, cytokines are the major local mediators of intercellular communications required to integrate the stimuli response in immune and inflammatory processes. Different cytokines have been associated with inflammatory diseases, with the clinical outcome partly determined by the balance between pro-inflammatory (i.e. IL1β, IL12, IL6, IFN-γ or TNF-α) and anti-inflammatory molecules (i.e. IL10 or TGF-β).

Chemokines are a subgroup of small chemotactic cytokines that have diverse roles in controlling leukocyte migration in health and disease (Koelink *et al.*, 2012). One of the most important chemokines is CXCL8, a chemokine produced by macrophages and epithelial cells that is an important inflammatory mediator due to its chemoattractant and angiogenic functions (Koelink *et al.*, 2012).

5.4. Cell adhesion molecules

Vascular endothelial cells mediate the interactions between blood and vessels and between blood and tissues, playing a key role in many important physiological and pathological processes (Ley *et al.*, 2007). The adhesion of circulating monocytes to vascular endothelial cells is a critical step in both inflammation and atherosclerosis (Cronstein and Weissmann, 1993) (Bevilacqua *et al.*, 1994). Endothelial cells characteristically respond to pro-inflammatory stimuli such as TNF-α, LPS and IL1β and recruit leukocytes by selectively expressing adhesion molecules on the surface, including VCAM-1, ICAM-1 and E-selectin, also known as endothelial-leukocyte adhesion molecule [ELAM]-1 (Ley *et al.*, 2007).

B. *Cymbopogon citratus* (DC.) Stapf

1. Botany

1.1. Taxonomic classification

Cymbopogon citratus (DC.) Stapf was firstly described as *Andropogon citratus* by De Candolle and then re-classified by Otto Stapf (Negrelle and Gomes, 2007). Its botanical classification is described in Table 1.1. The genus *Cymbopogon* belongs to the Poaceae family, also known as Gramineae, and includes around 30 species. The name of *Cymbopogon* derives from the greek words "kymbe" (boat) and "pogon" (beard), referring to the flower spike arrangement, while *citratus* derives from the ancient Latin, meaning lemon-scented leaves. Other scientific names could be used to refer this species, namely: *Andropogon ceriferus* Hack, *A. citratus* DC, *A. citratus* DC ex Nees, *A. citriodorum* Hort x Desf., *A. nardus* subsp. *ceriferus* (Hack) Hack, *A. roxburghii* Nees ex Steud., *A. schoenanthus* L. and *Cymbopogon nardus* subvar. *citratus* (DC.) Roberty. Commonly names include lemongrass; "capim-limão", "capim-cidreira" and "capim-cidrão" in Brazil; and "erva-príncipe" in Portugal. To note that other species receive the popular denomination identical to *C. citratus*, for example: *Melissa officinalis* L. (Labiatae) and *Lippia alba* N.E.Br (Verbenaceae) – both called "capim-cidreira" or "erva-cidreira", and *Killinga odoratta* Vahl (Cyperaceae) - called "capim-cheiroso", "capim-santo" or "erva- cidreira" (Negrelle and Gomes, 2007).

Table 1.1. Taxonomic classification of *C. citratus* (DC.) Stapf (Negrelle and Gomes, 2007)

KINGDOM	**PLANTAE**
Subkingdom	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)
Division	Magnoliophyta (flowering plants)
Class	Liliopsida (Monocotyledons)
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae (R.BR) Barnhart, 1895 (grass family)
Genus	*Cymbopogon* Sprengel, 1815
Species	*Cymbopogon citratus* (D.C. ex Nees) Stapf, 1906

1.2. Morphology and anatomy

C. citratus (Fig. 1.6A) is a native herb from southwest Asia, presently cultivated around the world, mainly in tropical and subtropical countries. The plant bears a lemon-like odor and a citrus taste. It is a perennial, rhizomatous and tufted grass that grows forming dense clumps of up to 3 m tall, with short rhizomes. Leaf sheaths glabrous, greenish inside; leaf blades glaucous, erect, 30–100 × 0.5–2 cm, both surfaces scabrid, base gradually narrowed, apex long acuminate; ligules are membranaceous or arid, 0.4-0.5 cm long, rounded or truncate. Inflorescences are erect, usually panicles 30-60 cm long and reddish to russet in color. Spikes are sessile, small, with canaliculated ventral side, 0.45-0.5 cm long and ciliated margins. Glumes are equal or sub-equal; the lower glume is lance-shaped, bicarinated, with bilobulated apex and acutely curved margins from the middle upwards; the upper glume is lanceolated, 0.43-0.45 cm long, usually 1-nervate. Sterile lemma is lanceolated, ciliated, 0.35 cm long and 2-nerved; fertile lemma is lineal, bifid, ciliated, 0.25 cm long and 1-nerved. Palea is lanceolated, 0.45 cm long and, usually, 1-nerved. Spikelets are pedicelled, 4–5 mm. Stamens 3. Ovary superior; styles 2; stigmas feathery. Fruit an oblong caryopsis (Negrelle and Gomes, 2007) (Fig. 1.6B-D).

The upper epidermis of *C. citratus* consists of a single layer of elongated cells interrupted by large bulliform cells, with thin walls and large vacuoles, which are involved in involution and folding of leaves (Eltahir and AbuEReish, 2010). The spongy parenchyma of *C. citratus* is formed of 1-2 cells thick following the upper epidermis (Fig. 1.6E). The vascular bundles, formed of about 3-4 layers of sclerenchyma, are embedded in chlorenchyma cells thus making a Kranz structure (Metcalfe, 1960). Positive results with $FeCl_3$ reagent (large dark cells in the section) revealed depositions of tannins in the walls of both abaxial and adaxial epidermal cells, being much more frequent in the abaxial surface. Additionally, staining with Sudan III demonstrated the presence of large quantities of oil in the leaf epidermal cells and in the mesophyll (Eltahir and AbuEReish, 2010).

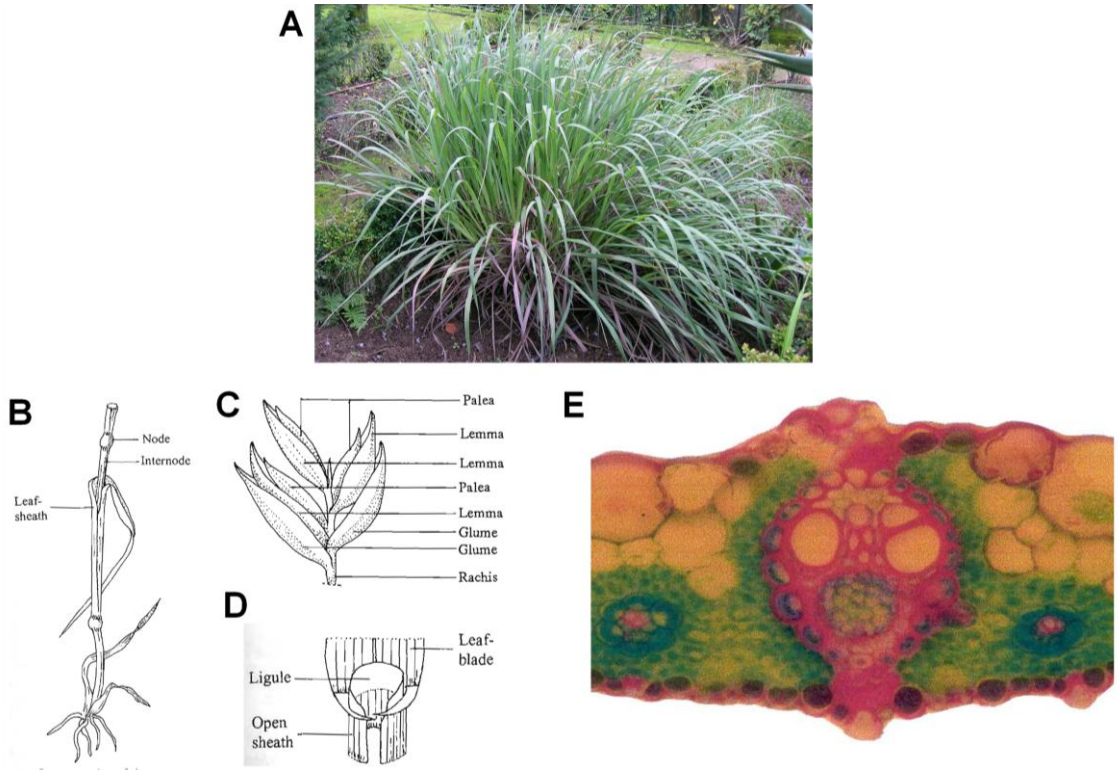


Figure 1.6. Cymbopogon citratus (DC.) Stapf (A), image kindly supplied by Mónica Zuzarte. Schematic representation of general strutures of Graminae family: (B) lower portion of shoot; (C) structure of a spikelet; (D) stem section and leaf-sheath. Transverse section of the leaves of *Cymbopogon citratus* (E). Adapted from Eltahir and AbuEReish, 2010; Hickey and King, 1997.

1.3. Propagation and culture

Cymbopogon citratus essential oil has a high economic value due to the widespread use in pharmaceutical, cosmetics, food flavor and agriculture industries (Negrelle and Gomes, 2007). Therefore, this plant is cultivated on a large scale, specifically in the tropics and subtropics, and several efforts have been made to optimize the plant culture conditions.

The propagation occurs by root or plant division because it rarely flowers or sets seed. It prefers a warm climate (24-26ºC) with well-distributed rainfall (average precipitation of 2500-2800 mm/year), altitudes bellow 500 m and a well-drained soil with pH between 5.5 and 7.5 (Oyen and Nguyen, 1998; Ortiz *et al.*, 2002). The culture of *C. citratus* is mainly made on hill slopes as a rainfed crop. The crop provides a maximum yield from the second to fourth year of planting and economic yield up to the fifth year; thereafter the yield declines considerably (Akhila, 2010). The addition of compost from palm oil mill sludge to a sandy soil improves the growth and the root development of the plant (Yaser *et al.*, 2007).

The quantity and the quality of essential oil is influenced by several genetic, agronomic and geoclimate factors (Akhila, 2010). In particular, oil content is lower during the month of heavy rainfall as compared to the dry months. The photoperiod, intensity of light, temperature and seasons or months of harvesting also influence the essential oil content and composition. Additionally, the superior-quality oil yield could be influenced by storage, drying, handling and, most importantly, harvesting (Akhila, 2010).

Temporary immersion system (TIS) has been used to favor the constant growth of the plantlets with rapid biomass increase. This system was created in the 80's by Harris and Mason, to reduce the handling time of the conventional method using semisolid (agar) medium (Etienne and Berthouly, 2002). In TIS, plant material is immersed in growth media for short periods, and at regular intervals that are sufficient for the plants to take up the nutrients. The main advantages of this system are a higher production of plants/m^2, reduced costs, manipulations and labor (semi-automated system), as well as improved plant quality. The highest multiplication coefficient, height, fresh and dry weight of *C. citratus* has been achieved with 4-6 immersions per day (Akhila, 2010). Standard conditions of TIS also favored the synthesis of secondary metabolites, namely flavonoids, which is correlated to the plant antioxidant activity (Tapia *et al.*, 2007).

2. Pharmacology

2.1. Ethnopharmacology

C. citratus has been used fresh, dried or powered for different applications such as, Caribbean and Asian cooking, fragrance in cosmetic products, relaxant in aromatherapy and, mostly important, as a healing herb. In traditional medicine, the plant has been used for the treatment of several inflammation-based pathologies as well as other health problems (Shah *et al.*, 2011). The recent ethnomedicine studies about *C. citratus* are summarized in Table 1.2. Its usual way of use is ingestion of an infusion made by pouring boiling water on fresh or dried leaves (which is called "abafado" in Portuguese). Decoction of leaves is also common. This plant is used in the folk medicine of several countries, being one of the most commonly used in Brazil. In general, it is used for the treatment of feverish conditions and disturbances of nervous and gastrointestinal systems, due to its antibacterial, antifungal, anxyolitic, hypnotic and anticonvulsant properties (Negrelle and Gomes, 2007; Shah *et al.*, 2011).

Table 1.2. Recent studies on ethnomedicine of *C. citratus* (DC.) Stapf.

COUNTRY	TYPE OF EXTRACT	USES	REFERENCE
Argentina	Infusion of leaf	Colds and cough	Hilgert, 2001
	Decoction of leaf	Stomachic, hypotensive and cardiotonic	Scarpa, 2004
	Leaf placed directly	Toothaches	
Bolivia	Decoction of leaf	Stomach pain, swellings by freezing cold and tranquilizer	Macía *et al.*, 2005
Brazil	Infusion of leaf and entire plant	Diarrhea, bad cold, headache, muscular pain, rheumatism, fever, hypertension, blood circulation, general pains and sedative	de Albuquerque *et al.*, 2007
	Decoction of leaf	Bad cold and rheumatism	Di Stasi *et al.*, 2002
	Infusion of root	Antidiuretic	
	Bath	Flu with scratching throat, witchcraft, envy and laziness	Coelho-Ferreira, 2009
China	Bath	Relieve pain	Au *et al.*, 2008
Democratic Republic of Congo	Decoction of leaf	Cough, stomach problems, diarrhea, fever, malaria, edema and to stimulate digestion	Mesia *et al.*, 2008
Cuba	Decoction	Nervousness, insomnia and fever	Cano and Volpato, 2004
Equador	Infusion of leaf	Gastritis, relaxant, stomach, pain and diarrhea	Tene *et al.*, 2007
Ethiopia	Root	Stomach-ache	Teklehaymanot *et al.*, 2007
Ghana	Poultice	Boils, swellings	Agyare *et al.*, 2009
French Guiana	Leaf in combination with other plants	Antimalarial	Vigneron *et al.*, 2005
Honduras	Decoction of leaf	Postpartum abdominal pain and lactation	Ticktin and Dalle, 2005
India	Oil of leaf	Carminative	Poonam and Singh, 2009
	Leaf	Stimulant, sudorific, antiperiodic and anticatarrhal	Khare, 2007
	Essential oil	Carminative, analgesic, antipyretic, antibacterial and antifungal	
Indonesia	Essential oil	Sedative, antiseptic and antiphlogistic	Zumsteg and Weckerle, 2007
Mexico	Infusion of aerial parts	Influenza and gastrointestinal disorders	Andrade-Cetto, 2009; Castillo-Juárez *et al.*, 2009
	Decoction of leaf	Cough	Giovannini and Heinrich, 2009

Table 1.2. (continued)

COUNTRY	TYPE OF EXTRACT	USES	REFERENCE
Nepal	The whole plant is pounded and then juice is taken with hot water	Cold	Shrestha and Dhillion, 2003
Nicaragua	Infusion of leaf	Abdominal and back pain, postpartum abdominal pain, promote lactation, fever, digestive ailments (flatulence, heartburn and stomach ache)	Coe, 2008
	Liniments of roots	Backache	
Nigeria	Decoction of leaf	Malaria	Ajibesin *et al.*, 2008; Dike *et al.*, 2012
		Yellow fever	Ajaiyeoba *et al.*, 2003
Peru	Leaf	Malaria and leishmaniasis	Kvist *et al.*, 2006
Portugal	Infusion of leaf	Gastric analgesic, digestive system, gall-bladder ailments, intestinal anti-inflammatory, sea-sickness, renal antispasmodic and bladder ailments	Novais *et al.*, 2004
Suriname	Bath of leaf	Baby´s abdominal pain, fever, cold and viral infections	Ruysschaert *et al.*, 2009
Thailand	Leaf or stem	Carminative, diuretic, pyreti	Wannissorn *et al.*, 2005
Uganda	Aqueous of leaf in combination with other plants	Malaria	Tabuti, 2008
	Leaf	Cold (Influenza)	Tabuti *et al.*, 2003

2.2. Bioactivity

Given the importance of *C. citratus* in traditional medicine, its biological relevance was extensively studied (Table 1.3). Aqueous extracts has been reported to have antimicrobial activity against bacteria and fungus (Okigbo and Mmeka, 2008). Ingestion of *C. citratus* infusion by human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) patients demonstrated therapeutical effects against oral candidiasis, this treatment being a good alternative to the typical treatment with gentian violet (Wright *et al.*, 2009). The antigenotoxic activity against gamma-rays has been also demonstrated (Fuentes *et al.*, 2006). Moreover, leaves infusion show *in vitro* anti-inflammatory properties through the inhibition of iNOS expression and NO production, in the LPS-stimulated dendritic cells (Figueirinha *et al.*, 2010), which could be related to the antioxidant activity of flavonoids and tannins (Figueirinha *et al.*, 2008). On the other hand, water-soluble polysaccharides enhanced T-lymphocyte proliferation, demonstrating immunostimulatory activity (Burana-Osot *et al.*, 2010). Additionally, studies in rats demonstrated the effectiveness of aqueous extracts in reducing cardiac rate and contractile force (Gazola *et al.*, 2004) as well as hypoglycemic and hypolipidemic effects (Adeneye and Agbaje, 2007).

Although aqueous extracts are the most used in folk medicine, the pharmacological activity of essential oil is the most studied, most likely due to its economical value. In general, essential oil is obtained by hydrodistillation and its properties include: antioxidant, anticarcinogenic, antimicrobial, anti-inflammatory, anti-ulcer, antipyretic, analgesic and antinociceptive (Table 1.3). In addition, it could be used for the treatment of nervous disturbances due to its ability to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), and to have antityrosinase, anxiolytic, sedative and anticonvulsant properties. Moreover, blood cholesterol is reduced after treatment of mice with essential oil. Additionally, the insecticide and repellent activities are largely reported. Furthermore, in agriculture, the essential oil is used as food preservative due to its action against insects and microorganisms that are responsible, for example, by stored-grain pests. Although not so common as essential oil, hydro-alcoholic extracts were also been studied and demonstrated similar activities (Table 1.3).

Table 1.3. Recent research on bioactivity of *C. citratus* (DC.) Stapf.

TYPE OF EXTRACT / ACTIVITY	REFERENCE
Aqueous	
Antibacterial against:	
Escherichia coli	Okigbo and Mmeka, 2008
Staphylococcus aureus	Okigbo and Mmeka, 2008
Antifungal against:	
Candida albicans	Okigbo and Mmeka, 2008
Antigenotoxic	Fuentes *et al*., 2006
Anti-inflammatory	Figueirinha *et al*., 2010
Antioxidant	Cheel *et al*., 2005; Figueirinha *et al*., 2008
Hypoglycemic and hypolipidemic	Adeneye and Agbaje, 2007
Immunostimulatory	Burana-Osot *et al*., 2010
Reduce cardiac rate and contractile force of heart	Gazola *et al*., 2004
Essential Oil	
Analgesic	Gbenou *et al*., 2012
Antibacterial against:	
Bacillus subtilis	Cimanga *et al*., 2002
Campylobacter jejuni	Wannissorn *et al*., 2005
Citrobacter sp.	Cimanga *et al*., 2002
Clostridium perfringens	Wannissorn *et al*., 2005
Enterococcus faecalis	Bassolé *et al*., 2011
Escherichia coli	Cimanga *et al*., 2002; Pereira *et al*., 2004; Wannissorn *et al*., 2005
Helicobacter pylori	Ohno *et al*., 2003
Klebsiella spp.	Cimanga *et al*., 2002; Pereira *et al*., 2004
Listeria spp.	Nguefack, Budde, *et al*., 2004; Viuda-Martos *et al*., 2010; Bassolé *et al*., 2011
Proteus spp.	Cimanga *et al*., 2002
Pseudomonas fluorescens	Tyagi and Malik, 2010a
Salmonella spp.	Cimanga *et al*., 2002; Wannissorn *et al*., 2005; Bassolé *et al*., 2011
Shigella spp	Cimanga *et al*., 2002; Bassolé *et al*., 2011
Staphylococcus aureus	Cimanga *et al*., 2002; Nguefack, Budde, *et al*., 2004
Anticarcinogen	Bidinotto *et al*., 2010
Anticonvulsant	Blanco *et al*., 2009; Silva *et al*., 2010

Table 1.3. (continued)

TYPE OF EXTRACT / ACTIVITY	REFERENCE
Essential oil	
Antifungal against:	
Aspergillus flavus	Mishra and Dubey, 1994; Paranagama *et al.*, 2003; Nguefack, Leth, *et al.*, 2004; Helal *et al.*, 2007; Singh *et al.*, 2010
Aspergillus spp. (other than flavus)	Nguefack, Leth, *et al.*, 2004; Bankole *et al.*, 2005; Pawar and Thaker, 2006; Nguefack *et al.*, 2009
Candida albicans	Abe *et al.*, 2003; Dutta *et al.*, 2007; Tyagi and Malik, 2010b; Xie *et al.*, 2012
Gibberella fujikuroi	Fandohan *et al.*, 2004; Nguefack, Leth, *et al.*, 2004
Penicillium spp.	Nguefack *et al.*, 2009
Saccharomyces cerevisae	Helal *et al.*, 2006
Anti-inflammatory	Gbenou *et al.*, 2012
Antinociceptive	Viana *et al.*, 2000
Antioxidant	Saeio, 2011
Antiparasite against:	
Bursaphelenchus xylophilus	Barbosa *et al.*, 2010
Leishmania spp.	Oliveira *et al.*, 2009; Santin *et al.*, 2009; Machado *et al.*, 2012
Plasmodium berghei	Tchoumbougnang *et al.*, 2005
Trypanosoma spp.	Santoro *et al.*, 2007; Habila *et al.*, 2010
Antipyretic	Gbenou *et al.*, 2012
Antityrosinase	Saeio, 2011
Anti-ulcer	Fernandes *et al.*, 2012
Anxiolytic	Blanco *et al.*, 2009; Costa, Kohn, *et al.*, 2011
Hypocholesterolemic	Costa, Bidinotto, *et al.*, 2011
Inhibition of AChE and BChE	Chaiyana *et al.*, 2010
Insecticide against:	
Aedes aegypti	Phasomkusolsil and Soonwera, 2011
Anopheles dirus	Phasomkusolsil and Soonwera, 2011
Culex quinquefasciatus	Pushpanathan *et al.*, 2006; Phasomkusolsil and Soonwera, 2011
Musca domestica L.	Kumar *et al.*, 2012
Sitophilus oryzae (L.)	Stefanazzi *et al.*, 2011
Tribolium castaneum (Herbst)	Stefanazzi *et al.*, 2011

Table 1.3. (continued)

TYPE OF EXTRACT / ACTIVITY	REFERENCE
Essential oil	
Repellent against:	
Anopheles darlingi	Moore *et al.*, 2007
Culex quinquefasciatus	Pushpanathan *et al.*, 2006
Sitophilus oryzae (L.)	Stefanazzi *et al.*, 2011
Tribolium castaneum (Herbst)	Olivero-Verbel *et al.*, 2010; Stefanazzi *et al.*, 2011
Sedative	Blanco *et al.*, 2009
Ethanolic	
Antibacterial against:	
Escherichia coli	Okigbo and Mmeka, 2008
Staphylococcus aureus	Okigbo and Mmeka, 2008
Anticarcinogen	Suaeyun *et al.*, 1997; Puatanachokchai *et al.*, 2002
Antifungal against:	
Candida albicans	Okigbo and Mmeka, 2008
Anti-inflammatory	Tiwari *et al.*, 2010
Antioxidant	Tiwari *et al.*, 2010; Koh *et al.*, 2012
Cardio- and hepato-protective	Gayathri *et al.*, 2011; Koh *et al.*, 2012
Inhibition of AChE and BChE	Khattak *et al.*, 2005
Methanolic	
Antigenotoxic	Rao *et al.*, 2009
Anti-inflammatory	Sforcin *et al.*, 2009; Bachiega and Sforcin, 2011
Antioxidant	Nakamura *et al.*, 2003; Rao *et al.*, 2009
Antispasmodic	Devi *et al.*, 2011
Insecticide and repellent against:	
Anopheles arabiensis	Karunamoorthi and Ilango, 2010; Karunamoorthi *et al.*, 2010
Vasorelaxant	Runnie *et al.*, 2004; Devi *et al.*, 2012

2.3. Toxicity

Considering the beneficial effect of *C. citratus* to human health and its suitability as pesticide for crop protection, with consequent potential on human health, evaluation of its adverse effects is an important parameter to take into account. A usual approach is the extrapolation from *in vitro* and *in vivo* laboratory models to evaluate the human health risks. Results of *C. citratus* toxicity, obtained from such models, are described below.

The cytotoxicity of the hydro-alcoholic extract has been evaluated in *Artemia salina* L., the brine shrimp larva, an invertebrate used to determine medium lethal concentration (LC50), a method with a good correlation (r = 0.85) to medium lethal dose (LD50) reported in mice (Lagarto Parra *et al.*, 2001). Using these approaches, the LC50 of *C. citratus* hydro-alcoholic extract was calculated as 9.83 μg/mL, while LD50 was infered as 460 mg/Kg, after oral administration.

The cytogenetic activity of aqueous extracts from leaves was evaluated in *Lactuca sativa* root tip meristem cells (Sousa *et al.*, 2010). Concentrations of 10; 20 and 30 mg/mL reduced the mitotic index, the seed germination and the root development, inducing chromosome aberrations and cellular death, while the dose of 5 mg/mL showed no toxicity. Another study analyzed the cytotoxic and genotoxic effects of *C. citratus* aqueous extract using *Allium cepa* assay (Akinboro and Bakare, 2007), demonstrating the mitodepressive effects on cell division and the induction of mitotic spindle disturbance and the 50% reduction of cell viability (EC50) was determined as 3% (v/v, of a *C. citratus* decoction prepared using 40g of plant/L). However, ethanolic extracts showed no genotoxic effect neither in *Aspergillus nidulans* (Ramos Ruiz *et al.*, 1996), at 1.23 mg/mL, neither in *Salmonella typhimurium* strains (Vinitketkumnuen *et al.*, 1994).

The cytotoxicity of essential oil was evaluated in the human epidermic cell line HaCaT by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Using this *in vitro* model, the concentration that caused an inhibition of 50% in cell viability (IC50) was achieved at 150 μL/mL (Koba *et al.*, 2008). The essential oil also demonstrated phototoxicity, a chemically induced skin irritation requiring ultraviolet radiation. Using 3T3 neutral red uptake phototoxicity assay, an effective concentration of the essential oil was achieved at 40–50 μg/mL (Nathalie *et al.*, 2006). The toxicity and genotoxicity of the essential oil was also investigated in murine models. In mice, the single LD50, based on a 24 h acute oral toxicity, was found to be around 3500 mg/kg, while a repeated-dose up to 100 mg/kg for 21 days did not demonstrate deleterious effects, thus indicating the safety of long-term treatment with essential oil (Costa, Bidinotto, *et al.*, 2011). In rats, a single oral

dose of 5000 mg/kg essential oil is not lethal but caused the reduction of food and water intake, thus evidencing a lethargic state, while repeated administration at 125–500 mg/kg/day did not significantly change their feeding pattern (Adeneye and Agbaje, 2007). Doses higher than 1500 mg/kg caused significant functional damages to stomach and liver of rat (Fandohan *et al.*, 2008).

In humans, it was reported that the contact of skin with *C. citratus* leaves could lead to contact dermatitis (Ross, 2010), which is problably due to essential oil and its components. Indeed, some essential oils constituents, namely citral, eugenol and geraniol, were identified as weak allergens in local lymph node assay (Lalko and Api, 2006).

3. Phytochemistry

The high pharmacological and economic value of *C. citratus* leaves prompted chemists to determine its chemical composition, that have allowed the identification of bioactive compounds and the establishment of chemical markers. The chemical constituents of extracts can vary depending on the stage of collection, parts of plant used, the plant origin and other factors. Therefore, chemical markers are important to: enhance product quality control; ensure the reliability and repeatability of pharmacological and clinical research; and to disclose the bioactivities and the possible side effects (Kamboj, 2012).

3.1. Analytical methods

Chemical composition and bioactivity of medicinal plants extracts depends on the nature of the extrated compounds. So, the choice of the extracting solvent and conditions is of utmost relevance and it might be inferred from the traditional uses.

Chromatographic methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) are widely used methods for extracts characterization. GC is normaly used for essential oils analysis and HPLC for phenolic compounds study, coupled to Ultra-violet/Visible and/or mass spectrometer detectors. Nuclear magnetic resonance (NMR) is also an important analytical tool.

3.1.1. Gas chromatography (GC)

GC, also known as gas liquid chromatography (GLC), is a technique for the separation of mixtures into components, that is based on the redistribution of the components between a stationary phase (in the form of a liquid, solid or combination of

both) and a gaseous mobile phase (Kamboj, 2012). GC can be a suitable technique to analyze volatile compounds present in essential oil of *C. citratus*. The identification of essential oil components has been done by comparing the retention index of the compounds in study with those of reference compounds, literature data or using computer matching with laboratory-made or commercial spectral libraries (Akhila, 2010). In order to avoid misleading identifications, the evaluation of retention indices on two columns of different polarity is highly recommended.

3.1.2. High performance liquid chromatography (HPLC)

Essentially, HPLC is a type of chromatography in which the stationary phase consists of small particles (3-50 μm) packed in a column with one end attached to a source of pressurized liquid eluent (mobile phase). The optimal separation by HPLC involves many factors like the composition of mobile phase, its pH and pump pressures (Kamboj, 2012). In the analysis of plant extracts, HPLC has been extensively used because it is easy to perform and not limited by the volatility or stability of the sample.

3.1.3. Mass spectrometry (MS)

MS, one of the most effective techniques to analyze complex samples, involves the measurement of the compound´s mass (m) as a function of charge (z), m/z, by 3 steps: ionization, mass analysis and detection.

Once the sample has been introduced into the mass spectrometer, it must be ionized. Ionization could take place by the impact of the compound of interest with electrons in a relatively energetic reaction that leads to a loss of an electron and the subsequent formation of a cation. Alternatively, the chemical ionization can be used, in which reagent gas ions react with the compound of interest to form protonated molecules. The use of a specific type of ionization depends of the type of compounds to be analyzed (Vermerris and Nicholson, 2006).

After ionization, the resulting ion is analyzed as a function of its mass-to-charge ratio and directed to the detector of the mass spectrometer that gathers the information to generate the mass spectrum. Usually, an abundance of 100% is considered to the most intense ion, termed the base peak, and the other ions in the mass spectrum are normalized to this intensity (Vermerris and Nicholson, 2006).

Tandem mass spectrometry (MS^2), in which multiple stages of mass analysis separation can be accomplished, provides significant information on compounds structure, in addition to their molecular weights. In this analytical technique, the ion of interest is

selected and given an excess of energy. The excited molecule can then undergo fragmentation, with the resulting ions providing structural information about the original precursor ion (Vermerris and Nicholson, 2006).

The main advantages of MS are its high sensitivity and specificity, as it is able to identify compounds with the same molecular weight but with different atom composition, and sometimes even to differentiate stereoisomeric compounds. Additionally, it is easy to couple MS with separation techniques, such as GC and HPLC (Rao, 2012).

3.1.4. Nuclear magnetic resonance (NMR)

NMR spectroscopy is a method that determines the resonance frequency of ^{1}H or ^{13}C nucleus in the molecule. ^{1}H and ^{13}C-NMR [1D] techniques are widely used in structural elucidation of compounds present in plant extracts, but they are superficial and sometimes unambiguous. Recently the 2D and 3D-NMR, namely the two-dimensional nuclear magnetic resonance spectroscopy (TOCSY) and correlation spectroscopy (COSY), are preferred (Kamboj, 2012)

Formely, NMR spectroscopy was used to a full structural characterization and, sometimes, to confirm the occurrence of a compond tentatively identified by chromatography and/or MS. Currently, the computerized comparison of the chemical shifts of signals in the NMR spectrum with those reference spectra compiled in a library, allows the identification of compounds without previous separation of the extract.

The information obtained by each of these analytical approaches is complementary and the chemical identification based only in one of them should be avoided. In the past two decades, the on-line combination of a chromatographic separation technique with a spectroscopic detection method has become the most important approach for the identification and/or confirmation of chemical composition of plant extracts. In this field, the combination of GC-MS and HPLC with UV-VIS or MS has become the preferred approach. Other analytical techniques like NMR can provide additional and/or complementary information on structure (Kamboj, 2012).

3.2. Essential oil

The chemical composition of *C. citratus* essential oil has been extensively studied and it could vary with geographical origin (Negrelle and Gomes, 2007). However, hydrocarbons terpenes, alcohols, ketones, esters and mainly aldehydes have been constantly detected (Gbenou *et al.*, 2012; Kumar *et al.*, 2012).

The predominant compound is citral (30-94%), a mixture of the aldehydes neral (cis-citral or citral B) and geranial (trans-citral or citral A), being the later the most abundant (Fig. 1.7). As an exception, *C. citratus* from Ethiopia contains geraniol (40%), the corresponding alcohol of geranial, as main compound, followed by citral (13%) and α-oxobisabolene (12%) (Abegaz *et al.*, 1983). Citral is responsible for the lemon smell and its high content justifies the commercial interest of essential oil, its quality being determined by citral content. Other aldehydes have been also reported, such as isocitral, valeric, citronelal, anisaldehyde, cinnamonaldehyde and salicyaldehyde (Negrelle and Gomes, 2007; Akhila, 2010).

Hydrocarbons have been identified in the essential oil, being myrcene and limonene the most abundant (Fig. 1.7) (Gbenou *et al.*, 2012; Kumar *et al.*, 2012). The compounds Z-*β* and E-*β*-ocimene, *a*-pinene, phellandrene, *a*- and *β*-caryophyllene and *a*-oxobisabolene were also reported. Among the several alcohols and esters identified are: geraniol, the most abundant, linalol, citronelol, neomenthol, menthol, nerol, farnesol and terpineol (Negrelle and Gomes, 2007). A reduction in geraniol content in contrast to an increase in neral and myrcene was observed under rust disease (Boruah *et al.*, 1995). Ketones, such as ionones and methylheptenone, and the esters geranil formate, laurate, linalil acetate, geranil acetate, among others, were also identified. The presence of alkaloids was reported; however further confirmation is needed (Negrelle and Gomes, 2007).

The relative amounts of myrcene and citral allows the identification of two different chemotypes of *C. citratus*. The East Indian have equal amounts of myrcene and citral, while the West Indian has little myrcene but high amount of citral (Blanco *et al.*, 2009).

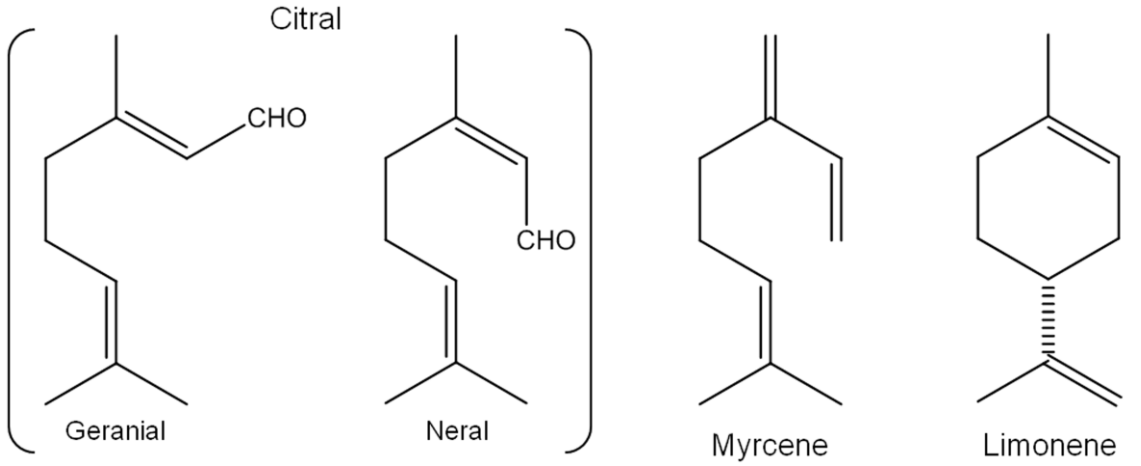


Figure 1.7. Structure of the main compounds present in the essential oil of *C. citratus* (DC.) Stapf.

3.3. Non-volatile compounds

The non-volatile components are less studied than the essential oil and include compounds with a great structural diversity. In the leaves, phenolic compounds, namely phenolic acids and flavonoids, have been the main compounds identified. The table 1.4 summarizes the most prevalent non-volatile compounds identified in the *C. citratus* leaves.

Phenolic acids, identified by several groups in the leaves of *C. citratus* (De Matouschek and Stahl-Biskup, 1991; Cheel *et al.*, 2005; Tapia *et al.*, 2007; Figueirinha *et al.*, 2008; Marques and Farah, 2009), include caffeic, *p*-coumaric and chlorogenic acids, as well its derivatives, and the isomers of caffeoylquinic, feruloylquinic and dicaffeoylquinic acids.

Among the flavonoids, *C*- and *O*- glycosides of luteolin and apigenin are prevalent. Aglycones have been also identified, but their presence is rare, compared to the glycosylated form (Gunasingh and Nagarajan, 1981; De Matouschek and Stahl-Biskup, 1991; Cagiotti and Etal, 2001; Miean and Mohamed, 2001; Cheel *et al.*, 2005; Tapia *et al.*, 2007; Figueirinha *et al.*, 2008). Glucose and rhamnose are the most frequent glycosides bonded to the aglycones. In general, the *C*-glycosylation occurs in C6 and C8 and *O*-glycosylation in C7. Recently, Figueirinha and collaborators (2008) have characterized the flavonoids from *C. citratus* leaves infusion and identified several glycosylated compounds, mainly *C*-glycosides of luteolin. The differences in the positions of glycosylation can be related with plant environmental conditions and/or with the extractive process.

Other non-volatile compounds, namely elemicin, catechol, hydroquinone, glutamic acid and proanthocyanidins have been also identified (Faruq and Etal, 1994; Tapia *et al.*, 2007; Figueirinha *et al.*, 2008).

Table 1.4. Non-volatile compounds identified in *Cymbopogon citratus* (DC.) Stapf leaves

CLASS OF COMPOUNDS	COMPOUNDS IDENTIFIED	REFERENCE
Phenolic acids	*p*-coumaric acid; caffeic acid	De Matouschek and Stahl-Biskup, 1991
	chlorogenic acid; caffeic acid	Cheel *et al.*, 2005
	caffeic acid; chlorogenic acid; neochlorogenic acid; *p*-hydroxybenzoic acid; *p*-hydroxybenzoic acid 3-*O*-*β*-D-glucoside	Tapia *et al.*, 2007
	3-, 4- and 5-caffeoylquinic acid; 3-, 4- and 5-feruloylquinic acid; 3,4-, 3,5- and 4,5-dicaffeoylquinic acid	Marques and Farah, 2009
	caffeic acid; caffeic and *p*-coumaric acid derivatives	Figueirinha *et al.*, 2008
Flavonoids	luteolin 6-*C*- and 7-*O*-glycosides	Gunasingh and Nagarajan, 1981
	luteolin 7-*O*-neohesperidoside; luteolin; isoorientin; orientin 2´´-*O*-rhamnoside	De Matouschek and Stahl-Biskup, 1991
	luteolin	Cagiotti and Etal, 2001
	apigenin; kaempferol; quercetin	Miean and Mohamed, 2001
	isoorientin; isoscoparin; swertiajaponin; isoorientin 2´´-*O*-rhamnoside; orientin	Cheel *et al.*, 2005
	isoorientin; 2´´-*O*-rhamnosyl isoorientin; 6-*C*-hexosyl-8-*C*-pentosyl luteolin; 6-*C*-pentosyl luteolin; 6-*C*-pentosyl-8-*C*-pentosyl luteolin; 6-*C*-pentosyl-8-*C*-deoxyhexosyl luteolin; 7-*O*-glucosyl luteolin; 7-*O*-neohes-perosyl luteolin; X´´-*O*-rhamnosyl *C*-pentosyl luteolin; X´´-*O*-rhamnosyl *C*-(6-deoxy-pento-hexos-ulosyl)	Figueirinha *et al.*, 2008
Others	elemicin; catechol; hydroquinone	Faruq and Etal, 1994
	glutamic acid	Tapia *et al.*, 2007
	proanthocyanidins	Figueirinha *et al.*, 2008

C. Phenolic compounds

Plants synthesize several secondary metabolites, many of them being phenolic compounds. These phytochemicals are structurally diverse and play several functions in the plant´s life cycle, which include: structural role in supporting or protecting tissues; defense from pathogens and insect pests (Vermerris and Nicholson, 2006); attractants for pollinators and seed-dispersing animals; allelopathic agents; protectors against ultra violet (UV); and signaling compounds in the interactions between plants and their environment (Fraga, 2010).

Secondary metabolites, besides their importance in plants, possess a high commercial significance to humankind as they have been used as dyes, fibers, glues, oils, waxes and flavoring agents, and are viewed as potential sources of insecticides, herbicides and new natural drugs (Fraga, 2010). In fact, many healthy effects of phenolic compounds have been reported, namely antioxidant, cardio- and neuro-protection, anti-cancer and anti-inflammatory (Stevenson and Hurst, 2007; González-Gallego *et al.*, 2010). Therefore, the study of phenolic compounds is extremely relevant for the search of new drugs, including anti-inflammatory agents.

1. Biosynthesis

Plant phenols are derived from the metabolism of carbohydrates, synthesized by photosynthesis from CO_2 and H_2O. Due to the high structural diversity of plant phenols, its biosynthesis is complex and involves several enzymes. There are two main biosynthetic pathways involved: the shikimate pathway and the malonate pathway (Vermerris and Nicholson, 2006) (Fig. 1.8).

The shikimate pathway results in the biosynthesis of chorismate, which can subsequently serve as a precursor for the biosynthesis of the aromatic amino acids tryptophan, phenylalanine and tyrosine (Tzin and Galili, 2010). Shikimate is synthesized from the substrates phosphoenolpyruvate and erythrose 4-phosphate, which are derived from the glycolysis and the pentose phosphate pathway, respectively, and is further converted to chorismate. Intermediates of shikimate pathway originates gallic acid that sterificates with glucose to yield *β*-glucogallin, the first specific metabolite in the route to hydrolyzable tannins (gallotannins and ellagitannins) (Ossipov *et al.*, 2003; Niemetz and Gross, 2005).

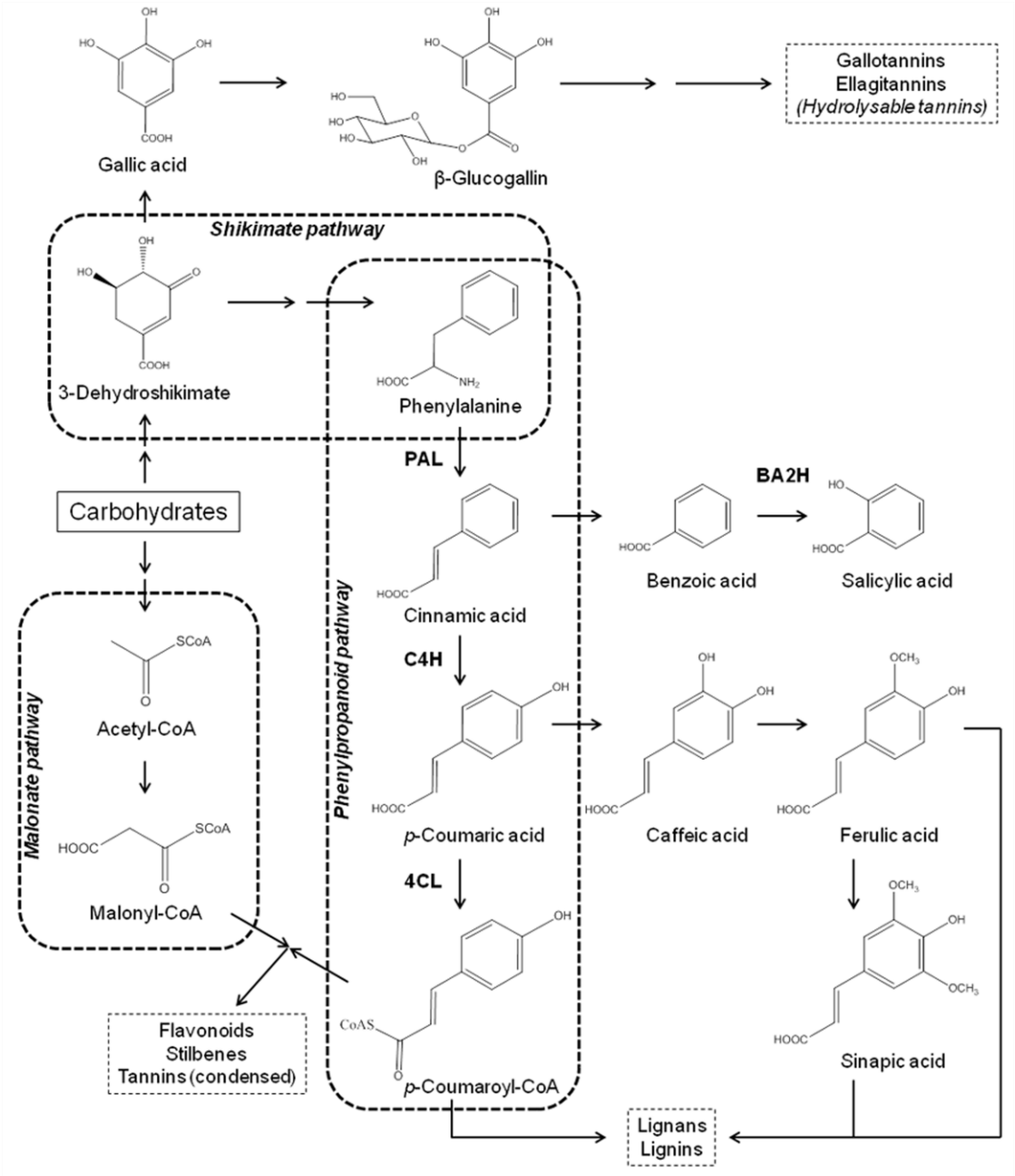


Figure 1.8. Schematic representation of the phenolic biosynthetic pathways. Enzyme abbreviations: PAL, phenylalanine ammonia lyase; BA2H, benzoic acid 2-hydroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, *p*-coumaric acid:CoA ligase. Adapted from Fraga, 2010.

Phenylalanine, originated from chorismate, is the precursor of phenolic acids, flavonoids and their derivatives by the phenylpropanoid pathway, in which the phenylalanine ammonia lyase (PAL) catalyses phenylalanine deamination, yielding cinnamic acid. Cinnamic acid is subsequently hydroxylated by cinnamic acid 4-hydroxylase (C4H) to

give *p*-coumaric acid. Then the enzyme 4-coumaric acid:CoA ligase (4CL) converts *p*-coumaric acid to *p*-coumaroyl Coenzyme A, the last metabolite of the pathway.

Further enzymatic reactions lead to the biosynthesis of specific classes of compounds (Vermerris and Nicholson, 2006). Cinnamic acid could be converted, by the benzoic acid 2-hydroxylase (BA2H), to benzoic acid and then to salicylic acid. The *p*-coumaric acid may be further hydroxylated and methylated leading to the formation of caffeic, ferulic and sinapic acids (Shahidi, 2004). Ferulic and sinapic acids, as well as *p*-coumaroyl CoA are the precursors of lignin and lignans (Ferrer *et al.*, 2008).

The flavonoids are formed via condensation of a *p*-coumaroyl CoA with three molecules of malonyl CoA (Fig. 1.9). The resulting chalcone can cyclize, in a reaction catalyzed by the chalcone isomerase (CHI), forming the flavanone naringenin. Flavanones have the basic skeleton of flavonoids (C6-C3-C6) and can originate other classes of flavonoids, namely flavones, isoflavones and anthocyanidins, depending on the substitution and unsaturation patterns (Shahidi, 2004).

Flavanone is converted by flavanone 3-hydroxylase (F3H) to yield dihydroflavonols, which can be converted by the flavonol synthase (FLS) to flavonols. Alternatively, the enzyme dihydroflavonol 4-reductase (DFR) converts dihydroflavonols to leucoanthocyanidins that are subsequently dehydrated by leucoanthocyanidin oxidase (LDOX). The forming anthocyanidins are subsequently glycosylated to yield anthocyanins (Vermerris and Nicholson, 2006). The enzyme DFR has been also reported to be involved in the proanthocyanidins biosynthesis through leucoanthocyanidin 4-reductase (LAR), which catalyses catechins formation. In addition, condensation of catechins yields proanthocyanidins, also named condensed tannins (Xie and Dixon, 2005).

Taking the basic skeleton of different flavonoid classes, hydroxylases, acyl-, methyl- and glycosyltransferases may synthesize a great diversity of flavonoids. In general, these types of substitution occur in the last biosynthetic steps (Ferrer *et al.*, 2008). In nature, the most common forms of flavonoids are the glycosylated ones. Glycosyltransferases transfer nucleotide-diphosphate activated sugars to the oxygen of hydroxyl group, the most common type of glycosylation, or to carbon atoms. More than a sugar unit could be conjugated, as well as different sugar types (Vogt and Jones, 2000).

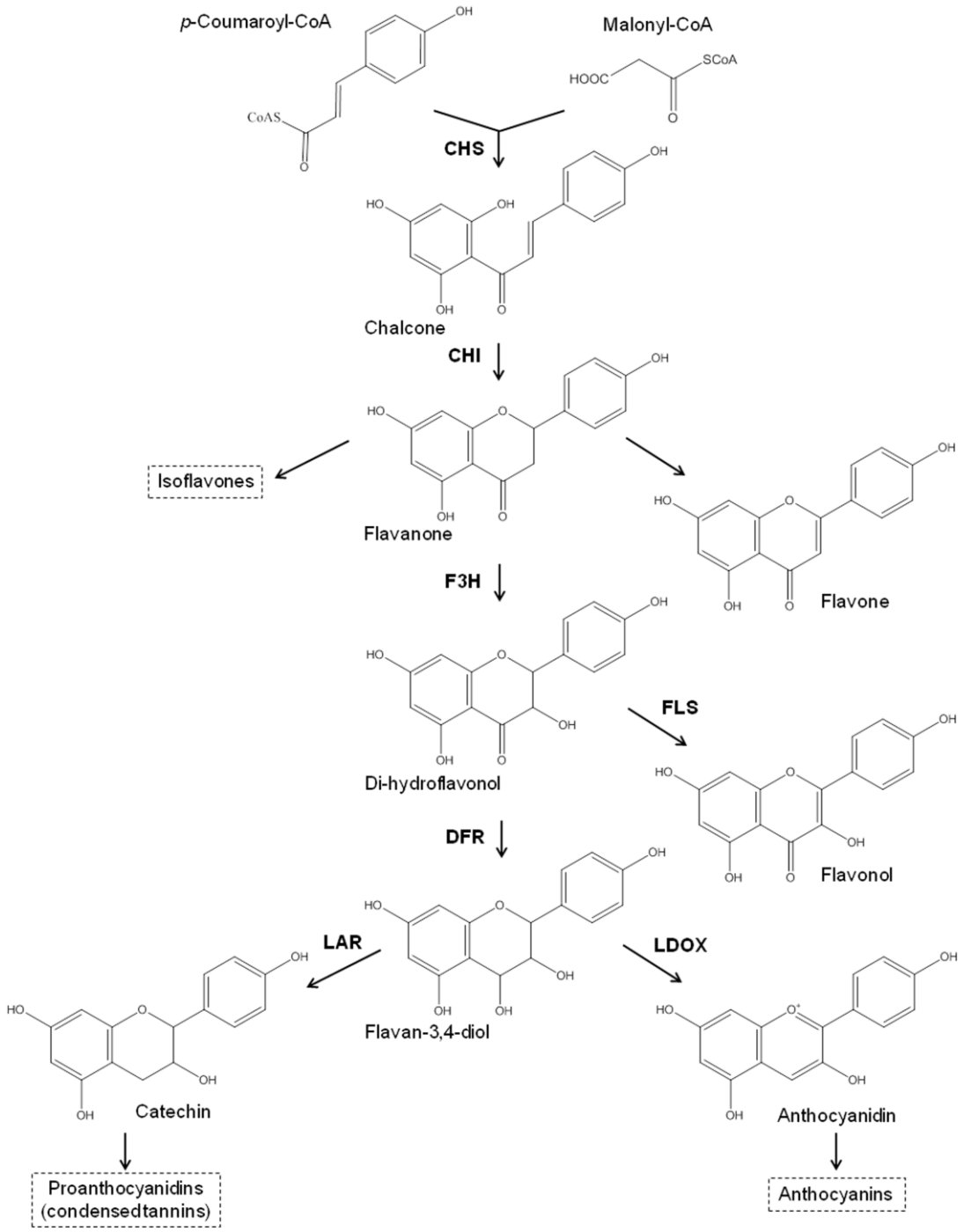


Figure 1.9. Schematic representation of the flavonoid biosynthetic pathway. Enzyme abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin 4-reductase; LDOX, leucoanthocyanidin oxidase. Adapted from Fraga, 2010

2. Classification, dietary sources and anti-inflammatory activity

Phenolic compounds are widely distributed in plants. Fruits, vegetables and beverages represent the main dietary sources, containing complex mixtures of phenolic compounds, with a large variety of structures. Given the structural diversity of phenols, it is difficult to chemically describe them. However, hydroxylated aromatic rings (phenol rings) are structural units present in all phenolic compounds. Therefore, they are divided into several classes according to the number of phenol rings and to the structural elements binding these rings to each other. The main groups of phenols are: phenol derivatives or simple phenols, phenolic acids, stilbenes, lignans, flavonoids, tannins, coumarins and anthraquinones (Fig. 1.10).

2.1. Simple Phenols

As derivatives of phenol and carbinol, simple phenols have properties of both combined. Tyrosol and hydroxytyrosol are the main simple phenols found in nature, being present in extra virgin olive oil and in some wines (Cabrini *et al.*, 2001; de la Torre *et al.*, 2006). As for their anti-inflammatory properties, simple phenols have been demonstrated to inhibit the binding of NF-kB and AP-1 transcription factors to the DNA, thus leading to a reduction of iNOS, COX-2, VCAM-1, ICAM and E-selectin expression (Carluccio *et al.*, 2003; De Stefano *et al.*, 2007). In addition, the inhibition of pro-inflammatory mediators NO, PGE_2 (Carluccio *et al.*, 2003), LTB_4 (Petroni *et al.*, 1997; de la Puerta *et al.*, 1999) and IL1β (Miles *et al.*, 2005), has been reported.

2.2. Phenolic Acids and Derivatives

Characterized by a phenol ring and a carboxylic function, they can be subdivided in two classes: benzoic acid derivatives (C6-C1) and cinnamic acid derivatives (C6-C3).

The hydroxybenzoic acids, such as gallic acid and protocatechuic acid (PCA), are found in very few plants consumed by humans, like blackberries, raspberries and tea (Shahidi, 2004). Despite the very low concentration of PCA in edible plants, Vitaglione and Donnarumma (2007) verified that the PCA concentration increases in the serum of human healthy volunteers after an anthocyanin-rich diet, suggesting that PCA could be considered the major anthocyanin metabolite *in vivo*. As for the anti-inflammatory properties, gallic acid is known to inhibit the AP-1 transcriptional activity (Maggi-Capeyron *et al.*, 2001) and PCA

increases the levels of Nfr2 by a mechanism mediated by the JNK pathway, leading to the induction of detoxifying genes (Varì *et al.*, 2011).

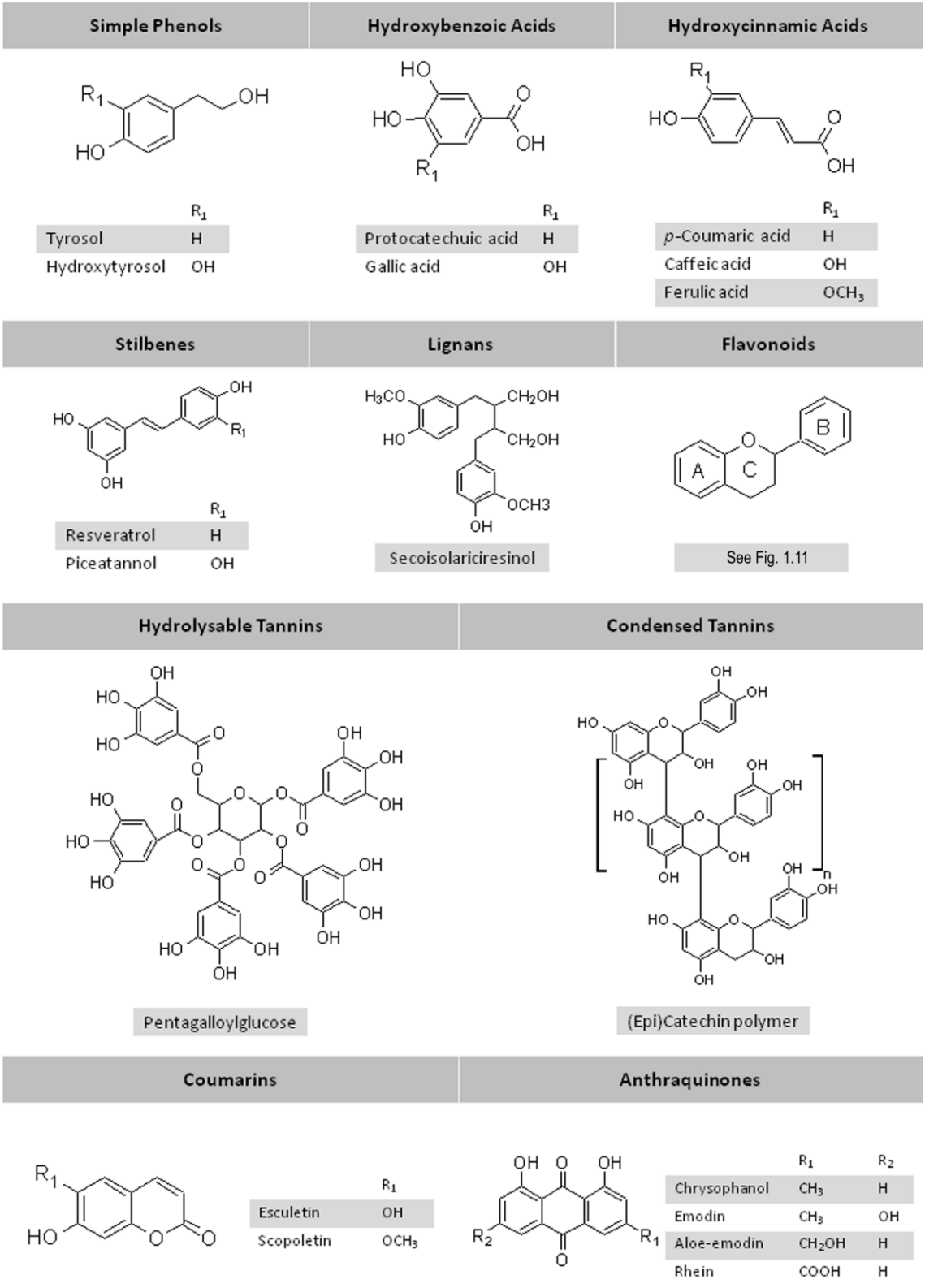


Figure 1.10. Chemical structures of phenolic compounds.

Hydroxycinnamic acids, trans-3-phenyl-2-propenoic acid derivatives differing in their ring substitution, comprise coumaric, caffeic and ferulic acids and their derivatives. Generally, they appear in glycoside forms or as quinic, shikimic or tartaric esters. For example, caffeic and quinic acids could combine to form chlorogenic acid, which is present in the coffee and many types of fruits, such as the kiwi (Clifford, 2000). Ferulic acid is present in cereal grains and, in opposition to caffeic acid, is a less abundant phenolic acid (D'Archivio *et al.*, 2007). As for the reported anti-inflammatory properties of caffeic acid, it inhibits the NF-kB and AP-1 activation (Maggi-Capeyron *et al.*, 2001; Moon *et al.*, 2009), the expression of VCAM, ICAM and E-selectin (Moon *et al.*, 2009), as well as the production of LTB_4, TNF-α, IL6, CXCL8 and CCL2 (De la Puerta *et al.*, 1999; Miles *et al.*, 2005; Moon *et al.*, 2009). Additionally, caffeic acid phenethyl ester (CAPE) has been reported to be a potent and specific activator of NF-kB (Natarajan *et al.*, 1996) and to induce the HO-1 expression through modulation of Keap1, Nrf2-ARE complex binding and p38 MAPK (Balogun *et al.*, 2003).

2.3. Stilbenes

Structurally, stilbenes are hydrocarbons with a *trans*-ethene double bond substituted with a phenyl group on both carbon atoms of the double bond. Stilbenes are present in low percentage in edible plants. Resveratrol and its isomer *trans*-resveratrol, mainly found in grapes and wine (Zhang *et al.*, 2006), are the most abundant molecules of this class. The anti-inflammatory properties of resveratrol include the inhibition of the activation of NF-kB and AP-1, which are involved in the reduction of iNOS and COX-2 expression, as well as CXCL8 production (Manna *et al.*, 2000; Donnelly *et al.*, 2004). In addition, the inhibition of the expression of adhesion molecules, such as VCAM-1, ICAM and E-selectin has been reported (Carluccio *et al.*, 2003). Furthermore, i*n vivo*, resveratrol also demonstrated anti-inflammatory activity in LPS-injected mice, as well as in colitis and atherosclerosis murine models (Martín *et al.*, 2004; Do *et al.*, 2008; Sebai *et al.*, 2009).

2.4. Lignans

Plant lignans contain a dibenzylbutane skeleton derived from phenylalanine through the dimerization of substituted cinnamic alcohols. Lignans are mostly found in their free form and linseed represents the main dietary source of these molecules (D'Archivio *et al.*, 2007). The lignan arctigenin has anti-inflammatory properties by inhibition of the

expression and activity of iNOS, as well as the production of NO, TNF-α and IL6 (Zhao *et al.*, 2009).

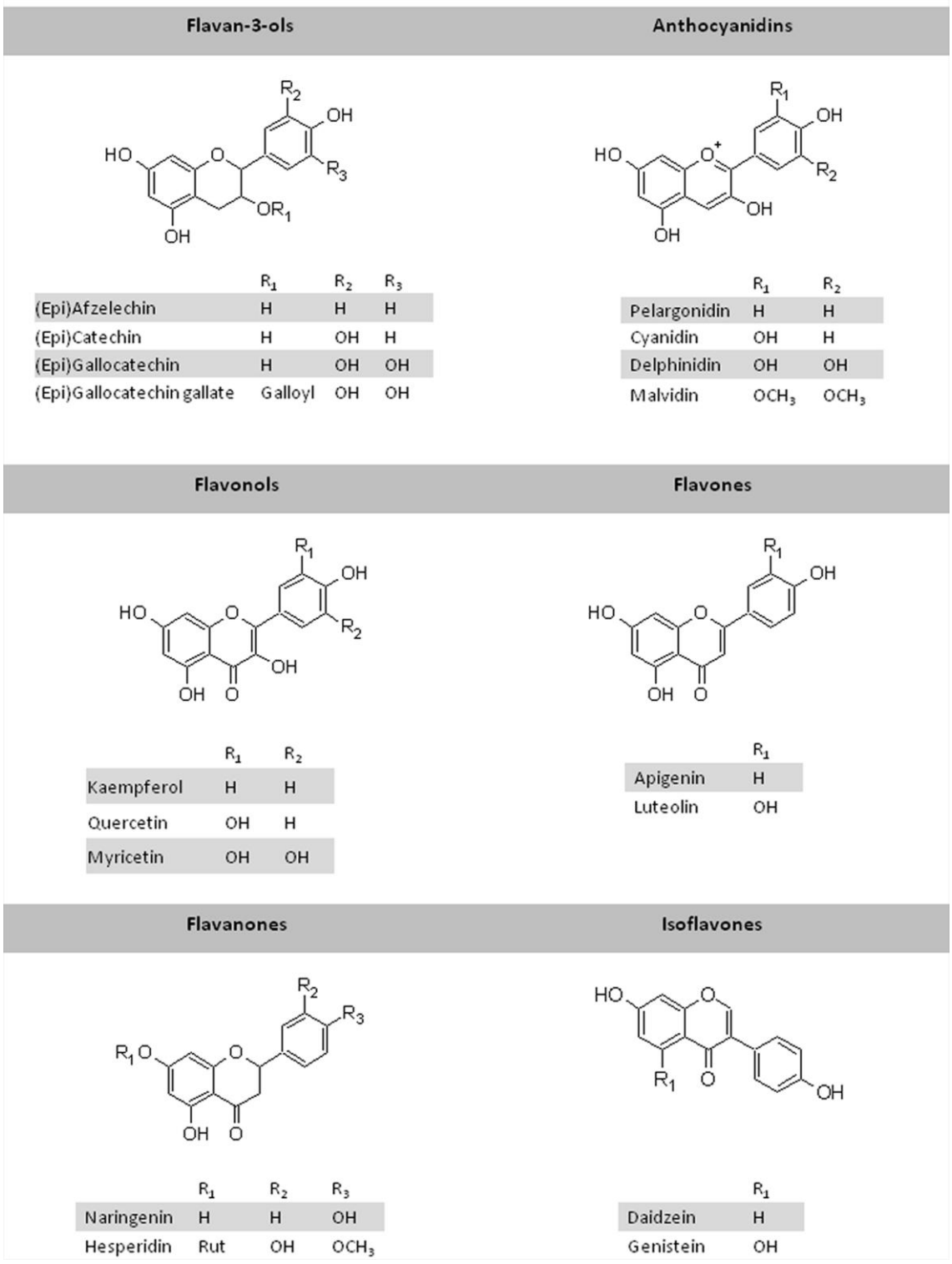
2.5. Flavonoids

Comprising more than 4000 naturally occurring compounds, flavonoids are nearly ubiquitous in the plant kingdom. These molecules have a great structural diversity, but they are all derived from a common biosynthetic pathway, the phenylpropanoid metabolic pathway, as described above. The basic structure of flavonoids is characterized by two aromatic rings (A and B) bonded to each other by 3 carbon atoms and 1 oxygen atom to form a third 6-member heterocyclic ring (C) (Beecher, 2003). Accordingly to the oxidation state and the functional group bonded to the C-ring, flavonoids are divided into subclasses. The main sub-classes of dietary flavonoids are flavan-3-ols, anthocyanidins, flavonols, flavones, flavanones and isoflavones (Fig. 1.11) (Gomes *et al.*, 2008; Crozier *et al.*, 2009).

Flavan-3-ols, also named catechins, are heterocyclic pyrans with no unsaturated bonds on the C-ring. Most flavonoids exist in nature predominantly as glycoside conjugates, with the exception of flavan-3-ols. Catechin, epicatechin and epigallocatechin gallate (EGCG) have been found as aglycones (molecule without any sugars bounded to it) in fruits, tea, red wine and cocoa products (Manach *et al.*, 2005). Catechins have been reported to show anti-inflammatory activity, like the inhibition of NF-kB and p38 MAPK activation (Ichikawa *et al.*, 2004; Mackenzie *et al.*, 2004; Kundu and Surh, 2007), iNOS expression (Liang *et al.*, 1999), COX-2 activity (Seeram *et al.*, 2003) and the production of cytokines, namely TNF-α, IL6 and IL12 (Ichikawa *et al.*, 2004; Mackenzie *et al.*, 2004). Additionally, EGCG induced the ARE-mediated transcriptional activity and showed *in vivo* anti-inflammatory properties in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated mice, in colitis and arthritis models, and in ischemic reperfused rat myocardium (Wei and Frenkel, 1993; Mackenzie *et al.*, 2004; Kundu and Surh, 2007; Abboud *et al.*, 2008; Morinobu *et al.*, 2008).

Anthocyanidins are also heterocyclic pyrans but with 2 double bonds and a positive charge in the C-ring. Cyanidin, malvidin, delphinidin and pelargonidin are present in berries and red wine, being responsible for their remarkable color (Manach *et al.*, 2005). The anthocyanidins glycosides, named anthocyanins, have been reported to inhibit the production of chemokines, namely CXCL8 and CCL2, and the expression of ICAM-1 (Youdim *et al.*, 2002). Cyanidin-3-*β*-glucoside inhibited NF-kB and ERK activation, iNOS

expression and cytokine production, namely TNF-α and IL6 (Pergola *et al.*, 2006; Zhang *et al.*, 2010).



	R_1	R_2	R_3
(Epi)Afzelechin	H	H	H
(Epi)Catechin	H	OH	H
(Epi)Gallocatechin	H	OH	OH
(Epi)Gallocatechin gallate	Galloyl	OH	OH

	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3

	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Myricetin	OH	OH

	R_1
Apigenin	H
Luteolin	OH

	R_1	R_2	R_3
Naringenin	H	H	OH
Hesperidin	Rut	OH	OCH_3

	R_1
Daidzein	H
Genistein	OH

Figure 1.11. Chemical structures of flavonoids.

Flavonols are pyrones with the carbonyl residue at C4, which contain one double bond C2=C3 and a hydroxyl group at C3. They are widely spread in vegetables, cereals, fruits, spices and herbs. Quercetin, myricetin and kaempferol represent flavonols significantly found in foods (Manach *et al.*, 2005). The anti-inflammatory properties of quercetin have been extensively studied. This flavonol inhibits the activity of NF-kB, AP-1, spleen tyrosine kinase (Syk), MAPKs, PI3K/Akt as well as the ubiquitin-proteasome system, and induced HO-1 and NQO1 expression (Shichijo *et al.*, 2003; Chen, Daniel, *et al.*, 2005; Chen, Ho, *et al.*, 2005; Tanigawa *et al.*, 2007; Marone *et al.*, 2008; Hwang *et al.*, 2009). Additionally, quercetin also showed inhibition of the expression of iNOS, COX-2, VCAM-1, ICAM-1 and E-selectin, as well as the production of NO, PGE_2, TNF-α, IL1β, IL6, CXCL8, CXCL2 and CCL5 (Choi *et al.*, 2004; De Stefano *et al.*, 2007; Min *et al.*, 2007). Moreover, quercetin demonstrated anti-inflammatory activity in LPS- or carrageenan-injected mice (Takahashi *et al.*, 2001; Shen *et al.*, 2002; Morikawa *et al.*, 2003). Myricetin has been reported to inhibit the activity of Syk and janus kinase (JAK)1/STAT3 (Shichijo *et al.*, 2003; Kumamoto *et al.*, 2009). Also kaempferol, has been shown to block NF-kB, AP-1 and JNK activation, Syk activity, iNOS, COX-2 and ICAM-1 expression, as wll as the production of NO and PGE_2 (Liang *et al.*, 1999; Chen *et al.*, 2004; Lee, Lee, *et al.*, 2010).

Flavones are characterized by one double bond C2=C3. Apigenin and luteolin may be found in edible vegetables, and also in spices and herbs. Both flavones have anti-inflammatory activity by inhibiting the activity of NF-kB, AP-1, Syk, MAPK and the proteasome. in addition, they have been reported to inhibit the expression of iNOS, COX-2, VCAM-1, ICAM-1 and E-selectin, as well as the production of NO, PGE_2, TNF-α, IL6 and CXCL8 (Gerritsen *et al.*, 1995; Kim *et al.*, 1999; Liang *et al.*, 1999; Xagorari *et al.*, 2002; Shichijo *et al.*, 2003; Chen *et al.*, 2004; Chen, Daniel, *et al.*, 2005). Apigenin is known to reduce the serum levels of immunoglobulin (Ig) E and IL4 in ovalbumin-sensitized mice (Yano *et al.*, 2007), while luteolin showed anti-inflammatory activity in LPS-induced lethal toxicity in mice (Kotanidou *et al.*, 2002).

Flavanones are pyrones with the carbonyl residue at C4 and without the double bond C2=C3. The most frequent flavanones are naringenin and hesperidin, which can be extracted from citrus fruits and juices (Manach *et al.*, 2005). Naringenin has been reported to have anti-inflammatory properties through inhibition of iNOS and COX-2 expression, as well as NO production (Chao *et al.*, 2010).

Isoflavones are characterized by one double bond C2=C3, but the connection of the B-ring is through the C3 of the C-ring instead of C2, as for other flavonoids. In general,

they are present in soybeans and other vegetables, genistein and daidzein being the most naturally occurring isoflavones. Genistein has been reported to inhibit the expression of iNOS and COX-2, and the production of NO, PGE_2, TNF-α and IL1β. It also induced the expression of PPARγ (Valles *et al.*, 2010). Furthermore, genistein inhibited inflammation and joint destruction in collagen-induced arthritis mice models (Verdrengh *et al.*, 2003).

Within the various classes, further differentiation is common, accordingly to the modifications of the basic structure: additional or reduced hydroxylation; methylation; isoprenylation; methylenation of ortho-dihydroxyl groups; dimerization to produce biflavonoids and bisulfates; and, mainly, the glycosylation of hydroxyl groups by hemiacetal bond to produce flavonoids *O*-glycosides or of the flavonoids core thus producing *C*-glycosides (Markham, 1982; Crozier *et al.*, 2009).

2.6. Tannins

Tannins are a group of water-soluble polyphenols with high molecular weight (500 to 3000 Da). This class of phenolic compounds is subdivided into hydrolysable and condensed tannins, as they are formed with phenolic acids units or 3-flavanols units, respectively. Mostly found in complexes with alkaloids, polysaccharides and proteins, tannins are generally present in fruits, vegetables, cereals, wine, tea and chocolate (Rangkadilok *et al.*, 2007). The tannins prodelphinidin-B4-3´-*O*-gallate, penta-*O*-galloyl-*β*-D-glucose and gallotannin have demonstrated anti-inflammatory properties by the inhibition of NF-kB activation, iNOS and COX-2 expression, as well as the production of NO, cytokines and chemokines (Lee *et al.*, 2003; Oh *et al.*, 2004; Erdèlyi *et al.*, 2005; Hou *et al.*, 2007). Also gallotannin is known to inhibit the activity of ubiquitin-proteasome system (Nam *et al.*, 2001).

2.7. Coumarins

Coumarins are lactones obtained by cyclization of hydroxycinnamic acids, belonging to the phenols with the basic skeleton of C6-C3. In general, they are characterized by great chemical diversity, mainly differing in the degree of oxygenation of their benzopyrane moiety. The major coumarins include simple hydroxylcoumarins (e.g. esculetin and scopoletin), furocoumarins and isofurocoumarins (e.g. psoralen and isopsoralen), pyranocoumarins (e.g. xanthyletin, xanthoxyletin, seselin and praeuptorin A), bicoumarins, dihydro-isocoumarins (e.g. bergenin) and others (e.g. wedelolactone). Herbs, fruits, vegetables, olive oil and beverages (coffee, wine and tea) are all dietary sources of

coumarins (Huang *et al.*, 2010). These phenolic compounds have been reported to inhibit the COX-2 and LO activity, with the consequent inhibition of PGE_2, TXB_2 and LTC_4 production (Hoult and Payá, 1996; Silván *et al.*, 1996; Resch *et al.*, 1998). In addition, they are known to inhibit the production of TNF-α, IL1β and IL6 (Kim, Jang, *et al.*, 2004).

2.8. Anthraquinones

Also known as hydroxyanthracenic compounds, anthraquinones are a group of colorful dyes common in plants, being very unstable and existing not only in several oxidation states but also bonded to sugars, mainly glucose and rhamnose. The most abundant anthraquinones are chrysophanol, emodins, aloins, rheins and senidins (Huang *et al.*, 2010). The reduced forms of anthraquinones (anthrones) may also dimerize to form dianthrones and these dimers suit as molecular skeletons to synthesize naftodianthrones (e.g. hypericin from *Hypericum perforatum*). Generally, anthraquinones can be found in distinct plant families such as Polygonaceae, Fabaceae, Asphodelaceae and Rhamnaceae, among others (Huang *et al.*, 2010). As for their anti-inflammatory properties, chrysophanol has been reported to inhibit NF-kB activation, COX-2 expression and the production of PGE_2, TNF-α and IL6 (Kim, Kim, *et al.*, 2010). In addition, emodin is known to inhibit NF-kB activation, the expression of iNOS, VCAM-1, ICAM-1 and E-selectin and the production of NO (Kumar *et al.*, 1998; Chen *et al.*, 2000).

In summary, phenolic compounds are widely distributed in plants and show anti-inflammatory activity by modulating the production and/or activity of inflammatory mediators, such as NO, PGE_2, cytokines and chemokines, due to their action on inflammatory signaling pathways namely: NF-kB, AP-1, PPAR and Nrf2 transcription factors; MAPKs; PTKs; PI3K/Akt and the ubiquitin-proteasome system. The identification and characterization of phenolic compounds that modulate signaling events involved in inflammation are important not only to validate the use of dietary phenols in human health but also in drug discovery, namely to find new potential molecules that serve as templates for further structural development of safe and effective anti-inflammatory drugs.

3. Bioavailability

Accurate information on the dietary intake of phenols is a difficult task because the content and profile of phenols present in food is affected by environmental factors, degree of ripeness, storage and culinary preparation, reason why only few estimations are available in the literature (Landete, 2012). But, even more important than knowing the amount of phenolic compounds present in the diet, it will be crucial to know how bioavailable is. Bioavailability referres to the concentration of a given compound or its metabolite at the target organ, and it depends on: the liberation of a compound from its matrix; absorption to the systemic circulation; distribution to the tissues; metabolism; and excretion via renal-, biliary- or pulmonary processes (Holst and Williamson, 2008).

The bioavailability of phenolic compounds has been extensively studied (Manach *et al.*, 2005; D'Archivio *et al.*, 2007; Crozier *et al.*, 2009). Generally, after oral intake, the phenol aglycones can be absorbed from the small intestine. However, most phenols are present in the food in form of esters, glycosides or polymers that cannot be absorbed in the native form. In these cases, compounds must be hydrolyzed by colonic microflora or intestinal enzymes before being absorbed. During the absorption, phenol aglycones are subjected to phase II metabolism within intestinal cells and later in the liver, thus forming sulfate, glucuronide and/or methylated metabolites. As a consequence, the forms present in the blood and tissues are different from those present in the food, so it is very difficult to identify the metabolites and to evaluate their biological activity. The chemical structure of polyphenols, more than their concentration, determines the rate and extent of their absorption, as well as the nature of the metabolites circulating in the plasma. In the blood, phenol metabolites circulate bounded to proteins, in particular albumin, what determines the rate of clearance and delivery to cells and tissues. Few studies have reported data on phenol concentrations in human and animal tissues, but it seems that their doses in the plasma and tissue are not directly correlated. At the cellular level, phenols can exert biological activities after deconjugation. The elimination of phenols and their derivatives occur mainly by urine and bile; extensively conjugated metabolites are primarily eliminated in the bile, whereas small conjugates are preferentially excreted in urine. In summary, the healthy effects of polyphenols depend on the amount consumed and, mostly, their bioavailability.

4. Structure-activity relationship

Structure-activity relationship constitutes a valuable information to develop new anti-inflammatory drugs from natural sources. Accordingly, the correlation between structure and biological activity of phenolic compounds, in particular flavonoids, has been elucidated in the last years. Table 1.5 summarizes the reports demonstrating the correlation between the anti-inflammatory properties of flavonoids and the presence of specific functional groups.

Flavonoids are anti-inflammatory agents in part due to their intrinsic antioxidant capacity. The scavenging of oxidizing species by flavonoids is related to the presence of the double bond C2=C3 in the C-ring of the flavonoid skeleton (Bonfili *et al.*, 2008). The inhibition of NO production and the expression of enzymes involved in the production of PGs and LTs, like PLA_2, 5- and 12-LOs, is also dependent on double bond C2=C3, having flavones with this double bond more activity than the homologous flavanones, which have a single bond at C2-C3 (Kim, Son, *et al.*, 2004). It was also demonstrated that the presence of a C2=C3 double bond in the C-ring is required for optimal inhibition of TNF-α-induced ICAM-1 expression by the luteolin (Benavente-García and Castillo, 2008).

The hydroxylation of the A-ring of flavonoids, in particular 5- and 7-hydroxylation(s) is important for the antioxidant capacity (Bonfili *et al.*, 2008), the inhibition of NO production (Kim *et al.*, 1999; Kim, Son, *et al.*, 2004) and the expression of cell adhesion molecules, such as ICAM-1 (Gerritsen *et al.*, 1995; Chen *et al.*, 2004). Additionally, the presence of hydroxyl group at the position 3 of the C-ring slightly blocks the inhibition of ICAM-1 expression by flavonoids (Chen *et al.*, 2004). Moreover, the inhibition of TNF-α production seems to require a 5,7-hydroxyflavone structure, which is common to apigenin and luteolin (Ueda *et al.*, 2004). Furthermore, the presence of 8-methoxyl group in the A-ring favors the inhibition of NO production (Kim *et al.*, 1999).

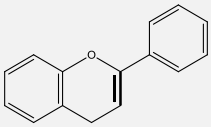
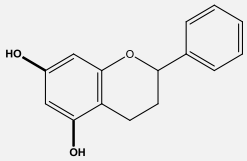
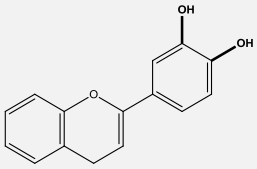
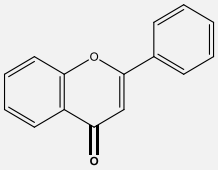
The flavonoids bioavailability correlates with their structure. In fact, the number of hydroxyl groups affects the affinity of flavonoids for the cellular membranes, influencing their structure, fluidity, permeability, as well as the intestinal absorption (Bonfili *et al.*, 2008). The potency of anti-inflammatory activity of flavonoids *in vivo* depends on the patterns and the number of hydroxyl groups on the B-ring. For example, 3',4'-dihydroxyl (catechol type) or 3',4'-hydroxyl/methoxyl (guaiacol type) groups are important for inhibition of granulomatic inflammation (Kim, Son, *et al.*, 2004). Also the 4´-OH substitution in the B-ring increases the potency of flavonoids in inhibiting the LPS-induced NO production and is important in ICAM-1 expression inhibitory properties of flavonoids

(Benavente-Garcia and Castillo, 2008). Finally, TNF-α inhibitory activity is promoted by the existence of the hydroxyl group in the C3' and C4' (B-ring), and their absence may reverse the flavonoids bioactivity (Ueda *et al.*, 2004). Furthermore, the presence of a carbonyl function at position 4 is required for the optimal inhibition of TNF-α-induced ICAM-1 expression, triggered by luteolin (Benavente-Garcia and Castillo, 2008).

Glycosylation also has an important role in the biological action of flavonoids. For example, flavonoid aglycones are more potent than the corresponding glycosides (diosmetin vs diosmin) (Gerritsen *et al.*, 1995; Benavente-Garcia and Castillo, 2008). The flavonoid glycosides may not penetrate the cell membrane due to their hydrophilicity, or to steric impediment caused by their large glycosyl residues (Kim *et al.*, 1999). However, the glucoside acetylation may help in the capacity of the compounds to suppress TNF-α expression (Shie *et al.*, 2010). Finally, the prenylated flavonoids that inhibit COX-2 activity have in common a C3 isoprenyl residue in their structure (Kim, Son, *et al.*, 2004).

The inhibition of proteasome activity by flavonoids strictly correlates with the number of hydroxyl groups on the B-ring and their methylation reduces this activity (Wan *et al.*, 2005; Bonfili *et al.*, 2008). It is also important to note that C4 could be a site of nucleophilic attack by the –OH group of N-terminal threonine of proteasomal subunit and that the C3 hydroxylation may alter the proteasome inhibitory activity of flavonoids (Chen, Daniel, *et al.*, 2005).

Table 1.5. Structure-activity relationship of flavonoids.

KEY FUNCTIONAL GROUP/ STRUCTURE	ANTI-INFLAMMATORY ACTIVITY (INHIBITION OF INDICATED ENZYMES AND INFLAMMATORY MEDIATORS)	REFERENCE
C-2,3-double bond 	NO production PLA_2 activity 5- and 12-LO activity ICAM-1 expression	(Kim, Son, *et al.*, 2004; Benavente-Garcia and Castillo, 2008; Bonfili *et al.*, 2008)
5,7-hydroxyls 	NO production TNF-α production ICAM-1 expression	(Gerritsen *et al.*, 1995; Kim *et al.*, 1999; Chen *et al.*, 2004; Kim, Son, *et al.*, 2004; Ueda *et al.*, 2004; Bonfili *et al.*, 2008)
hydroxyl in B-ring 	Proteasome activity Granulomatic inflammation TNF-α production ICAM-1 expression	(Kim, Son, *et al.*, 2004; Ueda *et al.*, 2004; Benavente-Garcia and Castillo, 2008; Bonfili *et al.*, 2008; Shie *et al.*, 2010)
4-carbonyl 	Proteasome activity ICAM-1 expression	(Chen, Daniel, *et al.*, 2005; Benavente-Garcia and Castillo, 2008)

D. Aims

Chronic inflammation is associated with several pathologies, including Alzheimer, diabetes, cardiovascular diseases, atherosclerosis and cancer, but current anti-inflammatory drugs are not totally safe and effective. Thus, the development of new anti-inflammatory drugs is of utmost importance. Additionally, *Cymbopogon citratus* (DC.) Stapf leaves infusion is used in traditional medicine for the treatment of inflammatory-related pathologies; however, little is known about its pharmacological activity, in particular its anti-inflammatory properties, as well as the action mechanisms and its bioactive compounds. Furthermore, phenolic compounds, which are secondary metabolites of plants with well known healthy effects, are promising molecules in the search of new anti-inflammatory drugs. Taking account all these points, the aims of the present study are:

▪ Evaluate the potential of *C. citratus* as source of new anti-inflammatory drugs, using *in vitro* bio-guided assays;

▪ Assess the contribution of phenolic compounds - phenolic acids, flavonoids and tannins - to the Cy activity;

▪ Elucidate the anti-inflammatory mechanisms of Cy and its phenolic compounds, by addressing in LPS-stimulated human and mouse macrophages:

- modulation of inflammatory mediators production, namely NO, PGE_2, cytokines and chemokines,
- modulation of intracellular signaling pathways activation, namely MAPKs, PI3K/Akt and NF-kB,
- modulation of proteasome activity;

▪ Further elucidate the Cy polyphenols chemistry, in order to establish a structure-activity relationship;

▪ Disclose the Cy bioactive compounds available to be incorporated in a pharmaceutical formulation with anti-inflammatory activity.

CHAPTER II

RESULTS AND DISCUSSION

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

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Vera Francisco, Artur Figueirinha, Bruno Miguel Neves, Carmen García-Rodríguez, Maria Celeste Lopes, Maria Teresa Cruz, Maria Teresa Batista. *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 as 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 LPS-stimulated RAW 264.7 macrophages as *in vitro* model. As inflammatory parameters, NO production was evaluated by Griess reaction, as well as effects on COX-2, 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 MAPK, JNK 1/2 and NF-kB. The ERK 1/2 and PI3K/Akt activation were not affected by Cy extract. Both phenolic acid- and tannin-rich fractions significantly inhibited NF-kB activation, iNOS expression and NO production but none of the PFs modulated MAPKs or PI3K/Akt activation. Neither Cy extract nor PFs affected LPS-induced COX-2 expression but LPS-induced PGE_2 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 drug.

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, diabesity (Schmidt and Duncan, 2003), cardiovascular and neurodegenerative diseases (Whitney *et al.*, 2009; Hunter and Doddi, 2010). 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 LPS, macrophages produce a variety of pro-inflammatory mediators, including PGE_2 and NO (Geller and Billiar, 1998). PGE_2 is synthesized by the rate limiting enzyme COX, while NO is synthesized by 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 express iNOS that produces high amounts of NO from l-arginine. To date, three isoforms of NOS have been identified: eNOS, nNOS and iNOS. The high-output of NO by iNOS contributes to the pathogenesis of septic shock and inflammatory diseases (Zamora *et al.*, 2000; Guzik *et al.*, 2003). 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, 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 MAPK pathways include p38 MAPK (Han *et al.*, 2010), JNK and ERK (Davis, 1994), and they are involved

on LPS-induced COX-2 and iNOS expression in macrophages (Chen and Wang, 1999; Chen *et al.*, 1999; Tsatsanis *et al.*, 2006). Accordingly, it has been demonstrated that MAPK inhibitors suppress the expression of iNOS gene (Chen *et al.*, 1999). Besides, the iNOS expression could also be modulated by PI3K/Akt pathway (Salh *et al.*, 1998), a serine/threonine kinase activated in response to certain growth factors and cytokines that provide a strong cell survival signal (Gold *et al.*, 1994; Crawley *et al.*, 1996). MAPKs and Akt also play a critical role in the activation of NF-kB (Nakano *et al.*, 1998; Carter *et al.*, 1999). The NF-kB 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-kB through several signaling pathways that lead to the phosphorylation of IkB by IKK, which is a marker for ubiquitination and subsequent degradation by proteasome. IkB degradation unmasks the nuclear localization motif of NF-kB, which is rapidly translocated to the nucleus, where it activates the transcription of target genes. Therefore, the involvement of MAPKs, Akt and NF-kB in the regulation of inflammatory mediator's synthesis makes them potential targets for novel anti-inflammatory therapeutics.

Cymbopogon citratus (DC.) Stapf, 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 and PGE_2 production as

well as activation of MAPKs, Akt and NF-kB 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 *Escherichia coli*.

2. Materials and methods

2.1. Materials

LPS from *E. 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, USA). Antibodies against phospho-p44/p42 MAPK (ERK1/2), phospho-p38 MAPK, phospho-SAPK/JNK 1/2, phospho-Akt (Ser473) and IkB-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 chemifluorescence 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* (DC.) Stapf were purchased from ERVITAL® (Mezio, Castro Daire, Portugal) in July 2004 and kept at −20ºC until use. 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. 2.1). Briefly, the extract was treated with water and fractionated on a reverse phase semipreparative column Lichroprep® RP-18 (310mm×25mm, particle sizes 40–63*μ*m), Merck (Darmstadt, Germany), eluted with water (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 (85cm×2.5 cm) using ethanol as mobile phase. All the fractionation process described above was monitored by HPLC and 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 (40ºC). The Cy extract and the polyphenol-rich fractions were weighted in sterilized and humidity-controlled conditions, and then solubilized in endotoxin-free water.

2.4. Cell culture

RAW 264.7, a mouse leukaemic monocyte macrophage cell line from American Type Culture Collection, and kindly supplied by Dr. Otília 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 *μ*g/ml streptomycin at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. 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 (6×10^5 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 *μ*g/ml LPS for 24 h. After the treatments, a MTT solution (5 mg/ml in phosphate buffered saline) was added and cells incubated at 37ºC for 15 min, in a

humidified atmosphere of 95% air and 5% CO_2. Supernatants were then removed and dark blue crystals of formazan solubilized with acidic isopropanol (0.04N HCl in isopropanol). Quantification of formazan was performed using an enzyme-linked immunosorbent assay (ELISA) automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

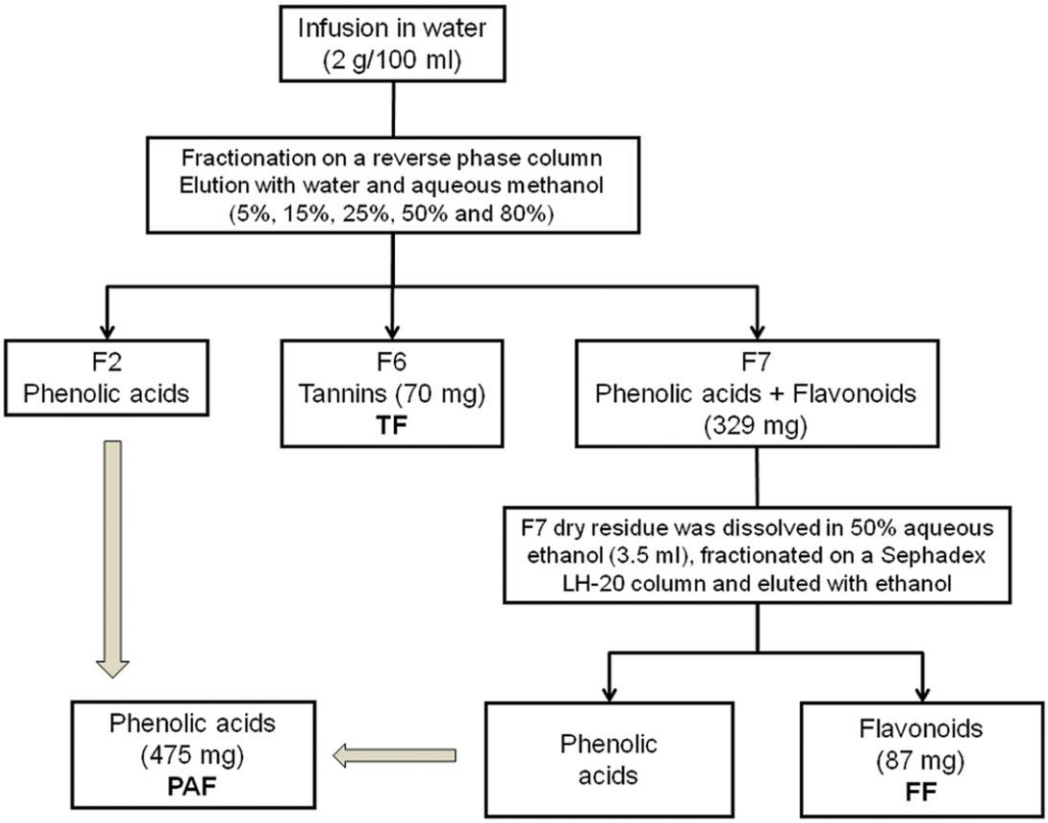

Figure 2.1. Fractionation scheme of *Cymbopogon citratus* (Cy) extract. Aqueous solution was fractionated on a reverse phase semi-preparative Lichroprep® RP-18 (310mm×25mm, particle sizes 40–63*μ*m) and Sephadex® LH-20 (85cm×2.5 cm) columns, providing three major fractions: phenolic acid-rich fraction (PAF), flavonoid-rich fraction (FF), and tannin-rich fraction (TF).

2.6. Measurement of nitrite production by Griess reagent

The production of 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 *μ*l 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) H_3PO_4] 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 PAF, 97.5 μg/ml FF, or 78 μg/ml 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 E_2 (PGE_2) by enzyme immunoassay (EIA)

To analyze the production of PGE_2, RAW 264.7 cells (6×10^5 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´s instructions.

2.9. Western blot analysis

To prepare total cell lysates for western blot analysis, RAW 264.7 cells (24×10^5 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 radio-immunoprecipitation assay (RIPA) buffer (50mM 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 (Sonics & Material INC.; Newtown, CT, USA) to decrease viscosity. The nuclei and the insoluble cell debris were removed by centrifugation at 4ºC, at 12,000×*g* 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-kB signaling pathways. Briefly, equivalent amounts

of protein were separated by 10% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. To examine the different proteins studied, the blots were incubated overnight at 4ºC with the respective primary antibodies: COX-2 (1:10,000), 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 IkB (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, Chalton St. Giles, UK). The generated signals were analyzed using the software ImageQuant TL® (GE Healthcare, Chalton St. Giles, UK). 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±standard error of the 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 analysis of variance (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 *C. citratus* (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 the anti-inflammatory potential and evaluated some molecular targets of Cy extract and its polyphenols 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 2.1).

Table 2.1. Effect of Cy extract, polyphenol-rich fractions and signaling pathways inhibitors on macrophage cell viability.

CONDITION	CELL VIABILITY (% OF CONTROL; MEAN ± SEM)
Control	100
Cy extract (1.115 mg/ml)	122.40 ± 3.71
PAF (530 μg/ml)	82.24 ± 3.95
FF (97.5 μg/ml)	84.69 ± 4.09
TF (78 μg/ml)	89.33 ± 5.80
LPS (1 μg/ml)	103.10 ± 5.46
LPS + Cy (1.115 mg/ml)	112.00 ± 5.59
LPS + PAF (530 μg/ml)	97.00 ± 7.15
LPS + FF (97.5 μg/ml)	92.12 ± 4.69
LPS + TF (78 μg/ml)	93.74 ± 5.73
LPS + Dexamethasone (20 μM)	96.97 ± 12.21
LPS + SB203580 (20 μM)	93.96 ± 9.08
LPS + SP600125 (20 μM)	95.58 ± 8.35
LPS + U0126 (10 μM)	89.68 ± 7.77
LPS + Wortmannin (500 nM)	86.06 ± 11.37
LPS + BAY 11-7083 (250 nM)	101.20 ± 5.88
LPS + Aminoguanidine (50 μM)	95.18 ± 8.21

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. 2.2A). In non-stimulated RAW 264.7 cells (control), COX-2 protein was almost undetectable, but after LPS treatment the expression strongly increased to 10,573±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. 2.2B, the cells treatment with LPS induced a great increase in PGE_2 production, consistent with the results obtained for COX-2 expression. However, PGE_2 production was 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 LPS to 7.56±0.29% of LPS). 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.

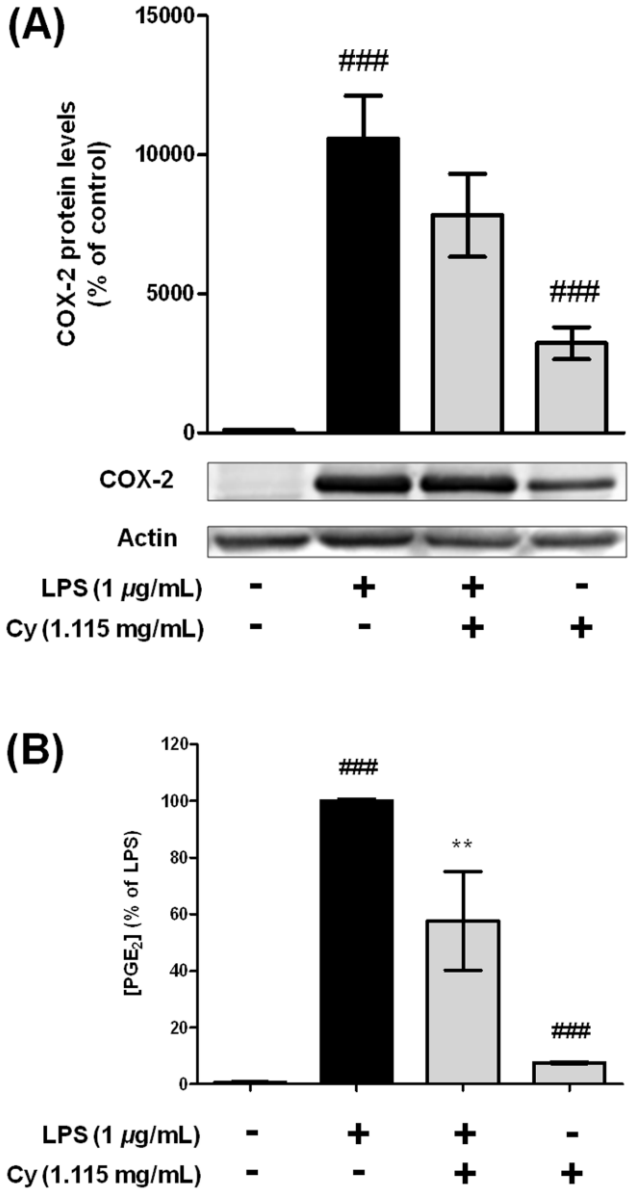

Figure 2.2. Lack of effect of *Cymbopogon citratus* (Cy) extract on LPS-induced COX-2 expression and inhibition of LPS-induced PGE_2 production in murine macrophages. RAW 264.7 cells were maintained in culture medium or pre-incubated with Cy extract for 1 h, and then treated with LPS for 24 h. (A) COX-2 expression was analyzed by western blot. An anti-actin antibody was used to confirm equal protein loading. The blot shown is representative of 3 blots yielding similar results. (B) PGE_2 levels were evaluated in the culture supernatants by enzyme immunoassay. Each value represents the mean±SEM from 2 to 3 independent experiments ($^{###}p < 0.001$, compared to control; $^{**}p < 0.01$, 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. 2.3A). 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. 2.3B, 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 has 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.

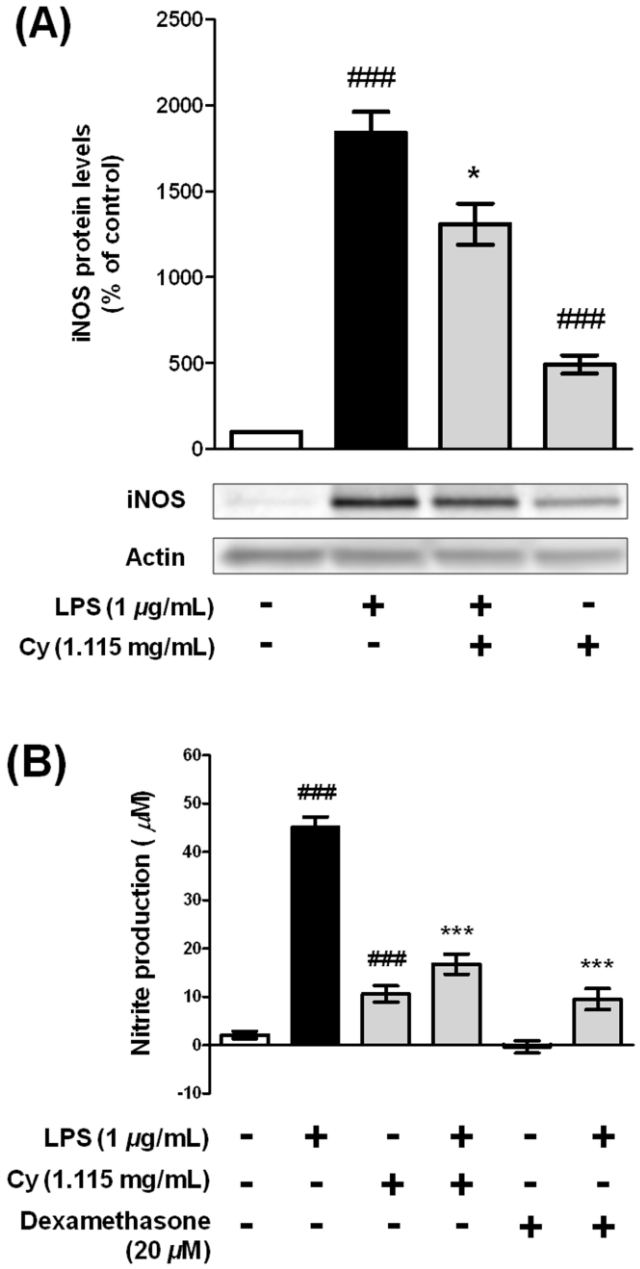

Figure 2.3. Inhibitory effect of *Cymbopogon citratus* (Cy) extract on LPS-induced iNOS protein expression and nitrite production in murine macrophages. RAW 264.7 cells were maintained in culture medium, or pre-incubated with Cy extract or dexamethasone for 1 h, and then treated with LPS for 24 h. (A) 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. (B) Nitrite levels in the culture supernatants were evaluated by the Griess reaction. Each value represents the mean±SEM from at least 3 experiments ($^{###}p < 0.001$, 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 2.1). As shown in Fig. 2.4, 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-kB 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.

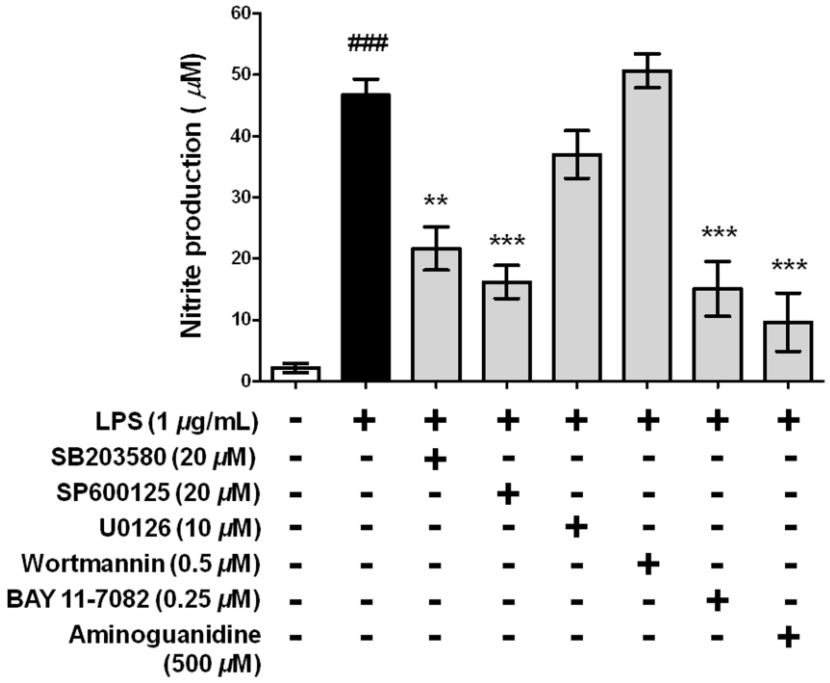

Figure 2.4. Evaluation of signaling pathways involved in the modulation of nitrite production on LPS-stimulated macrophages. RAW 264.7 cells were maintained in culture medium, or pre-incubated with the indicated inhibitors, and then LPS was added for 24 h. Nitrite levels in the culture supernatants were evaluated by the Griess reaction. Each value represents the mean±SEM from at least 3 experiments ($^{###}p < 0.001$, compared to control; $^{***}p < 0.001$, compared to LPS).

3.1.3. Cy extract inhibits LPS-induced activation of p38 MAPK, JNK 1/2 and NF-kB

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-kB 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. 2.5, 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-kB 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-kB activation is affected by the Cy extract in LPS-activated macrophages was carried out measuring IkBα proteolytic degradation by western blot. After 15 min of macrophages stimulation with LPS, we observed that IkBα was almost completely degradated (Fig. 2.5E). Pre-treatment of 1 h with 1.115 mg/ml Cy extract partially prevented the IkBα degradation induced by LPS and therefore the NF-kB activation. Taken together these data suggest that Cy extract selectively inhibits different LPS-induced pro-inflammatory signaling cascades.

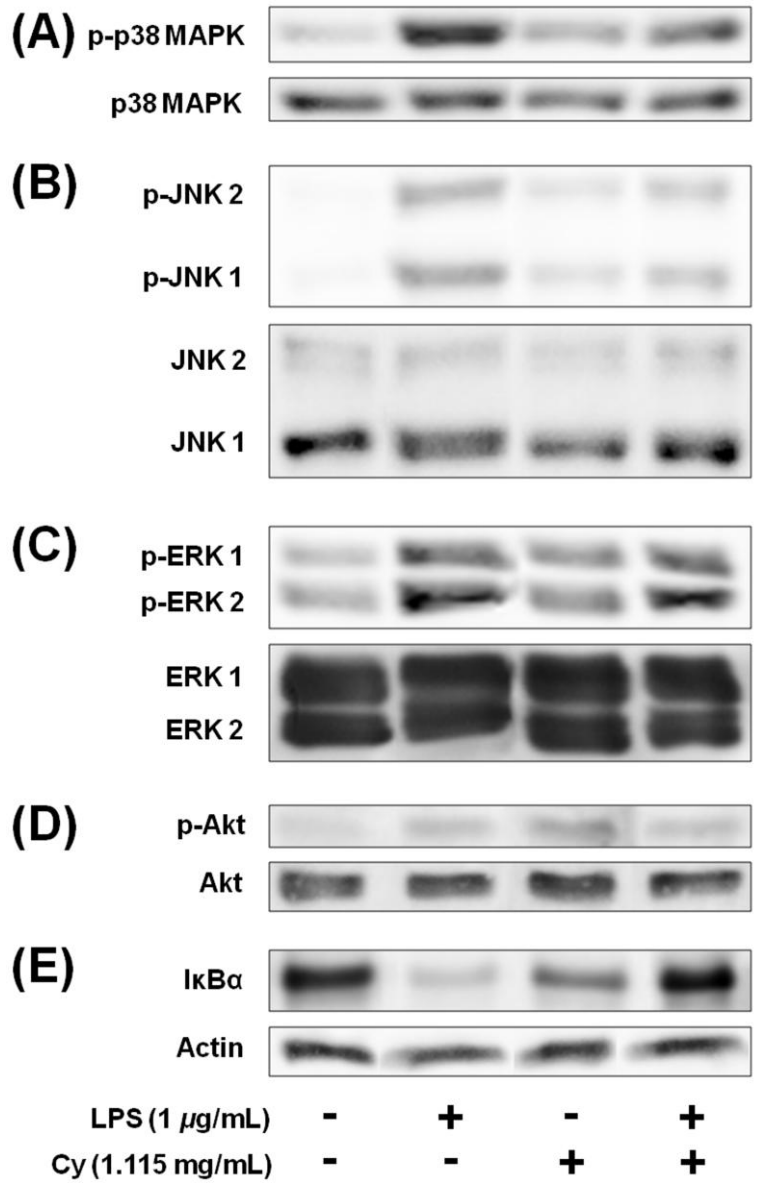

Figure 2.5. Inhibitory effect of *Cymbopogon citratus* (Cy) extract on the LPS-activation of p38 MAPK, JNK 1/2 and NF-kB signaling pathways. RAW 264.7 cells were maintained in culture medium, or pre-incubated with Cy extract for 1 h, and then treated with LPS for 30 min to see the effect on MAPKs and Akt phosphorylation, or for 15 min to see the effect on IkBα 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) IkBα 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, flavonoids and tannins, 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 cytotoxicity (Table 2.1) and on 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 don´t affect COX-2 expression, but PAF inhibits PGE_2 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. 2.6A).

Since Cy extract inhibited the PGE_2 production in LPS stimulated macrophages, we next investigated the contribution of polyphenolic fractions to this activity. As shown in Fig. 2.6B, the LPS-induced PGE_2 production is strongly reduced by PAF to 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 PGE_2 production.

The effect of Cy fractions on COX-2 expression and PGE_2 production in non-stimulated cells was also tested and none of the treatments interfered neither with the COX-2 expression nor with the PGE_2 production.

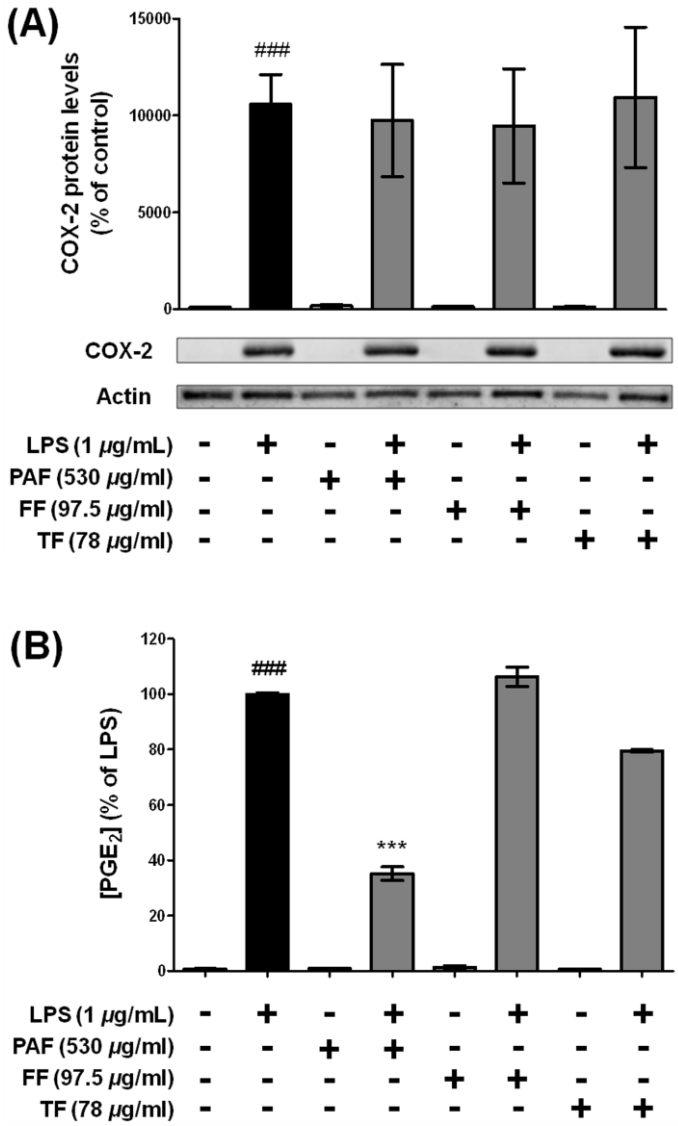

Figure 2.6. Lack of effect of polyphenol-rich fractions from *Cymbopogon citratus* (Cy) on LPS-induced COX-2 expression and inhibition of LPS-induced PGE_2 production by phenolic acid-rich fraction in macrophages. RAW 264.7 were maintained in culture medium or pre-incubated for 1 h with phenolic acid-rich fraction (PAF), or flavonoid-rich fraction (FF), or tannin-rich fraction (TF), and then treated with LPS for 24 h. (A) 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. (B) PGE_2 levels were evaluated in the culture supernatants by enzyme immunoassay. Each value represents the mean±SEM from 2 to 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. 2.7A) 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. 2.3A). In addition, PAF and TF fractions significantly inhibited the LPS-induced nitrite production by 50.63% and 41.59%, respectively (Fig. 2.7B). 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.

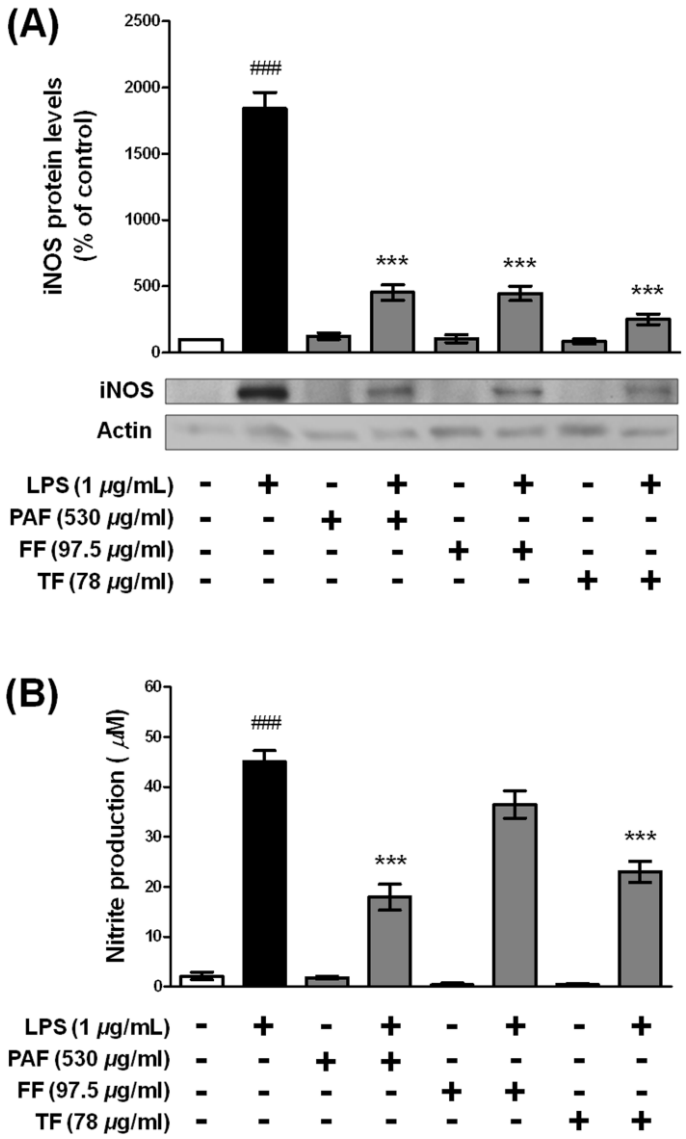

Figure 2.7. Inhibitory effect of polyphenol-rich fractions from *Cymbopogon citratus* (Cy) on LPS-induced iNOS expression and nitrite production. RAW 264.7 cells were maintained in culture medium or pre-incubated for 1 h with phenolic acid-rich fraction (PAF), or flavonoid-rich fraction (FF), or tannin-rich fraction (TF), and then treated with LPS for 24 h. (A) 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. (B) Nitrite levels in the culture supernatants were evaluated by the Griess reaction. Each value represents the mean±SEM from at least 3 experiments ($^{###}p < 0.001$, compared to control; $***p < 0.001$, compared to LPS).

3.2.3. Polyphenol-rich fractions inhibit LPS-mediated NF-kB 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. 2.8, 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-kB activation.

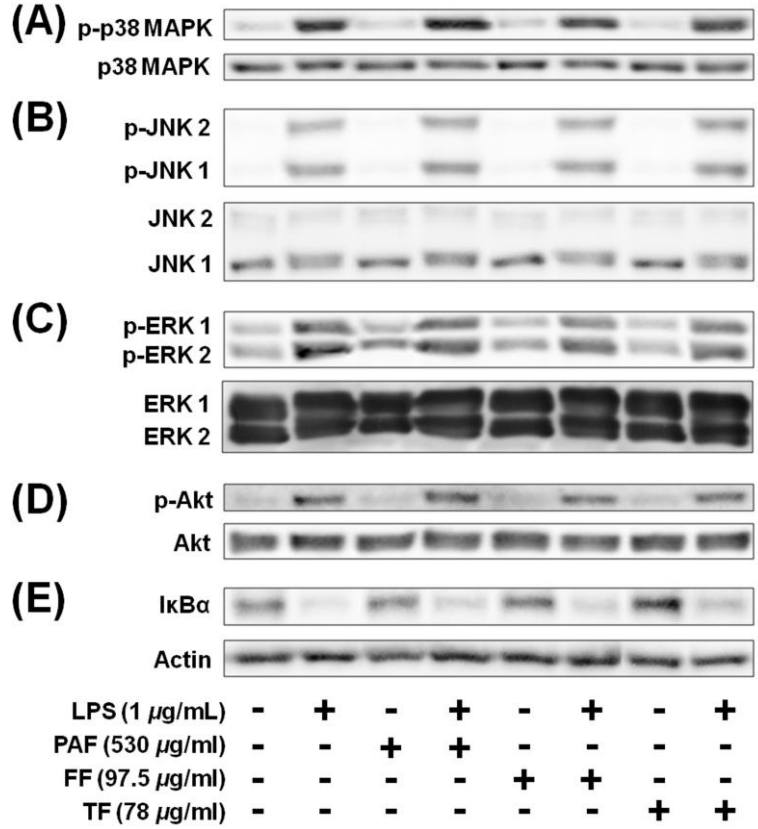

Figure 2.8. Inhibitory effect of polyphenol-rich fractions from *Cymbopogon citratus* (Cy) on LPS-activation of NF-kB signaling pathway. RAW 264.7 cells were maintained in culture medium, or pre-incubated for 1 h with phenolic acid-rich fraction (PAF), or flavonoid-rich fraction (FF), or tannin-rich fraction (TF), and then treated with LPS for 30 min to see the effect on MAPKs and Akt phosphorylation, or for 15 min to see the effect on IkBα 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) IkBα 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* 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-kB, 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 pathway inhibitors, it was observed that the LPS-induced NO production is mainly controlled by p38 MAPK, JNK 1/2 and NF-kB pathways. Accordingly, previous studies demonstrated that JNK 1/2 (Zhou *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 implicated in NF-kB activation (Nakano *et al.*, 1998; Carter *et al.*, 1999), being NF-kB 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-kB signaling pathways. Therefore, and since the signaling pathways involved in NO production are the same that Cy extract inhibited, the inhibition of NF-kB, 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, emphasizing its potent anti-inflammatory capacity and indicating that Cy extract inhibited NO production in part by inhibiting 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 (González-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-kB 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; Park *et al.*, 2008; Zhou *et al.*, 2008; Lee, Lim, *et al.*, 2010). 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 IkB degradation, suggesting that the inhibitory effect of the fractions on LPS-induced iNOS expression is due to inhibition of NF-kB activation, as described for other compounds

(Pan *et al.*, 2000; Cheng *et al.*, 2001). Since NF-kB 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-kB by Cy extract and to identify the compounds responsible for this effect, using bio-guided assays.

In the present study it was shown that Cy extract inhibited PGE_2 production, being phenolic acid-rich fraction (PAF) responsible for 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 on 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 nor 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 through 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-kB 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-kB signaling pathways in murine macrophages (Fig. 2.9), being the phenolic acids and tannins responsible for its anti-inflammatory properties through inhibition of transcription factor NF-kB, 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 a new anti-inflammatory drug 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.

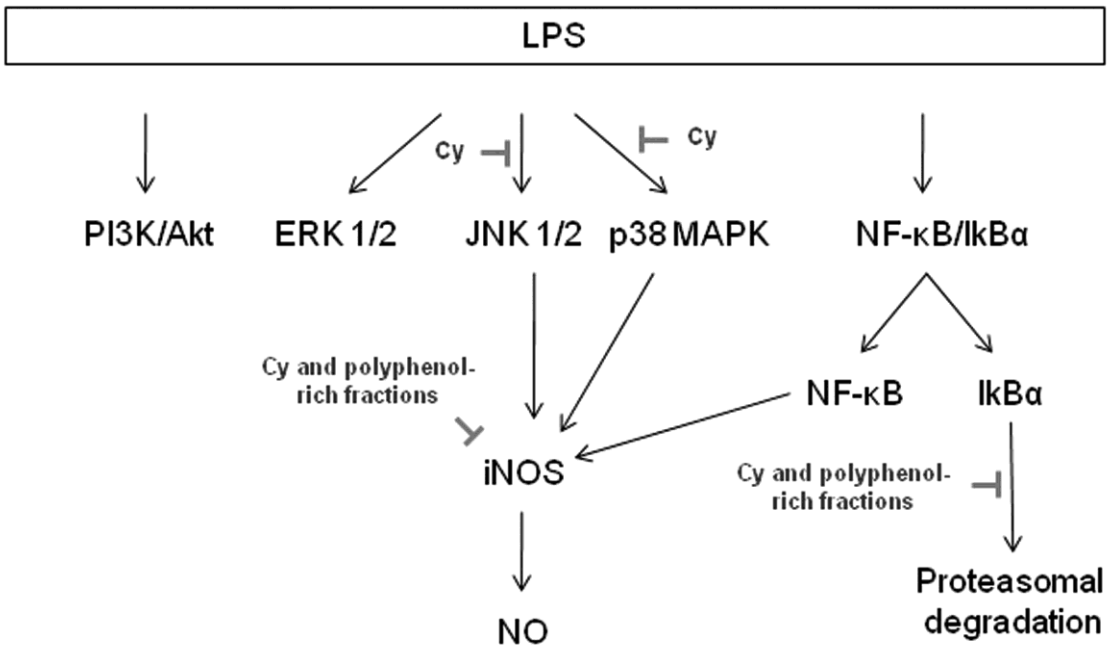

Figure 2.9. Schematic model for the anti-inflammatory mechanism of lipid- and essential oil-free infusion of *Cymbopogon citratus* leaves (Cy extract) and its polyphenol-rich fractions on LPS-stimulated murine macrophages.

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

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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-kB pathway inhibition: contribution of chlorogenic acid. Published in Journal of Ethnopharmacology 148 (2013), 126-34

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 bioactive compounds.

Aim of the study: Investigate the compounds responsible for anti-inflammatory potential of 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 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 LPS-stimulated human macrophages. Activation of NF-kB, a master regulator of inflammation, was investigated by western blot and gene reporter assay. Proteasome activity was assessed using a fluorogenic peptide.

Results: C. 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 NF-kB pathway, the results pointed to an inhibition of LPS-induced NF-kB activation by Cy and PFs. Chlorogenic acid (CGA) 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-kB 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-kB pathway and cytokine expression. Additionally, Cy polyphenols, in particular chlorogenic acid, were highlighted as bioactive compounds.

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 NF-kB by signaling events that lead to the phosphorylation of the IkB by the 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 IkB degradation unmasks the nuclear localization motif of NF-kB, allowing its rapid translocation to the nucleus and the transcription of many inflammatory mediators, like TNF-α and 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-kB 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-kB and proteasome pathways are good candidates for attenuating inflammation.

Cymbopogon citratus (DC.) Stapf, 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 NO production, in dendritic cells and mouse macrophages, through modulation of p38 MAPK, JNK 1/2 and NF-kB 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 LPS-induced cytokines production, in human macrophages, and the involvement of NF-kB and proteasome in the anti-inflammatory profile of *C. 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* (DC.) Stapf 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 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 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 minutes) and other isocratic elution for 5 minutes, at a flow rate of 200 μL/min. 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 the indicated concentrations of CGA (Sigma Chemical Co., St. Louis, MO, USA) for 1 h. Then, 1 μg/mL LPS from *Escherichia coli* (serotype 026:B6) (Sigma Chemical Co., St. Louis, MO, USA) was added. For cytokine expression, LPS was added by 24h; for western blot, LPS was added by 10, 15 or 30 min; for dual-luciferase assay, LPS was added by 8h; and for chymotrypsin-like activity of proteasome, LPS was added by 30 min. 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.; Wilmington, DE, USA). RNA reverse transcription was performed using iScript™ select cDNA synthesis kit (BioRad, Hercules, CA, USA), accordingly to manufacturer´s instructions, on C1000™ 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 2.2). Gene expression changes were analyzed using the built-in iQ5 Optical system software v2, with the Pfaffl method (Pfaffl, 2001). Gene expression was expressed as relative fold changes compared to LPS and normalized to GAPDH.

Table 2.2. 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.

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 manufacturer´s instructions. Protein concentration of cell lysates was determined by the bicinchoninic acid protein assay. Cell lysates were denaturated at 95ºC, 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-PAGE transferred to polyvinylidene fluoride membranes and specific antibodies (Cell Signaling Technologies, Danvers, MA, USA) against phospho-IkBα, total IkBα and NF-kB 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, Chalfont St. Giles, UK) and analyzed by software ImageQuant TL® (GE Healthcare, Chalfont St. Giles, UK). 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-kB-dependent firefly luciferase-expressing plasmid using Lipofectamine™ LTX and Plus Reagent (Invitrogen, Paisley, UK). 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. 2.10A). 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. 2.10B); 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, 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. 2.10A), 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 prepared from Cy extract, CGA being the most representative (Fig. 2.10C). 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.

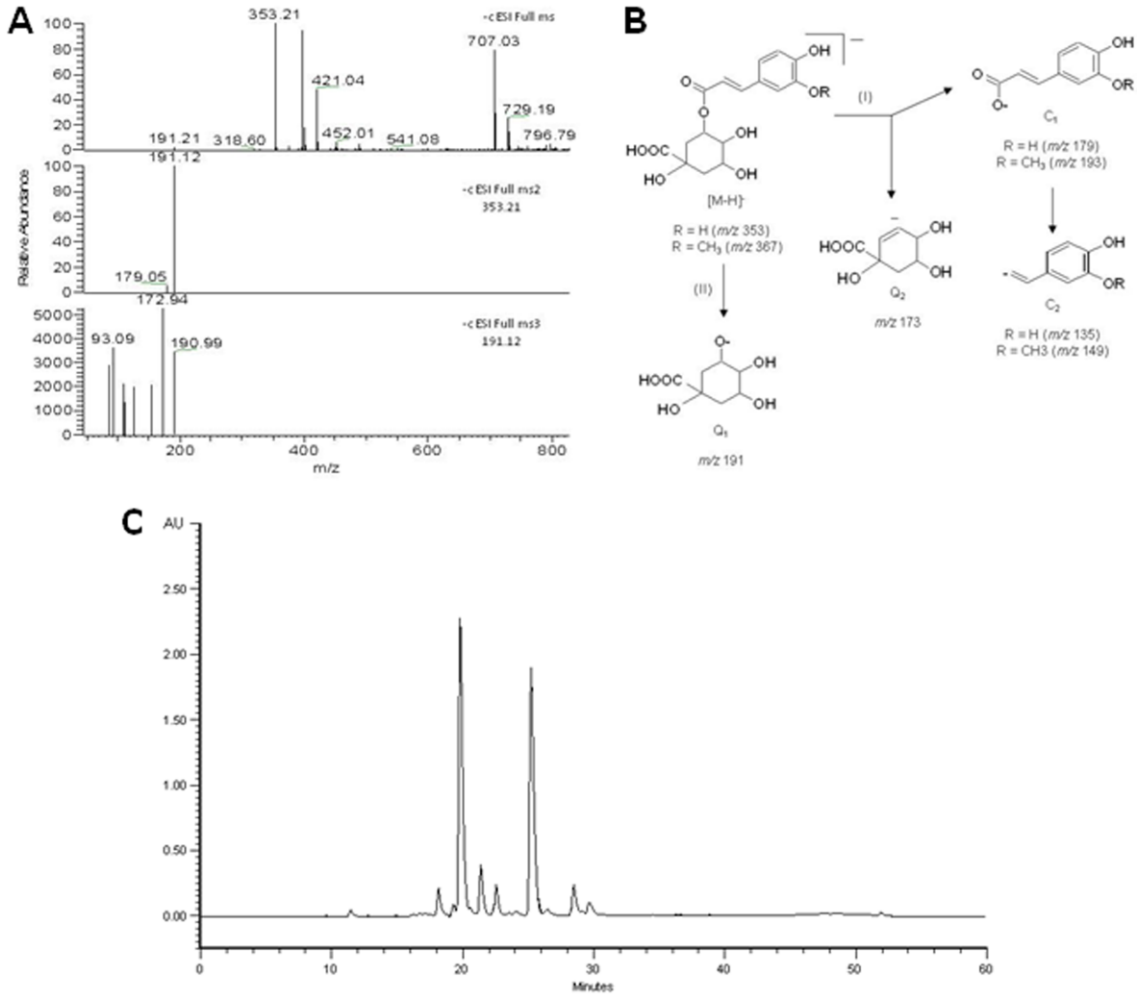

Figure 2.10. Chlorogenic acid of *Cymbopogon citratus* leaves. (A) Mass spectra. (B) Fragmentation pattern for hydroxycinnamic esters of quinic acid. (C) HPLC profile from phenolic acid-rich fraction, recorded at 320 nm, illustrating two main phenolic acids, namely chlorogenic acid (Rt=19.95 min) and *p*-coumaric acid (Rt=25.33 min), on conditions previously reported (Figueirinha *et al.*, 2008).

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. 2.11). 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 *C. citratus* and its polyphenols through the inhibition of cytokines expression.

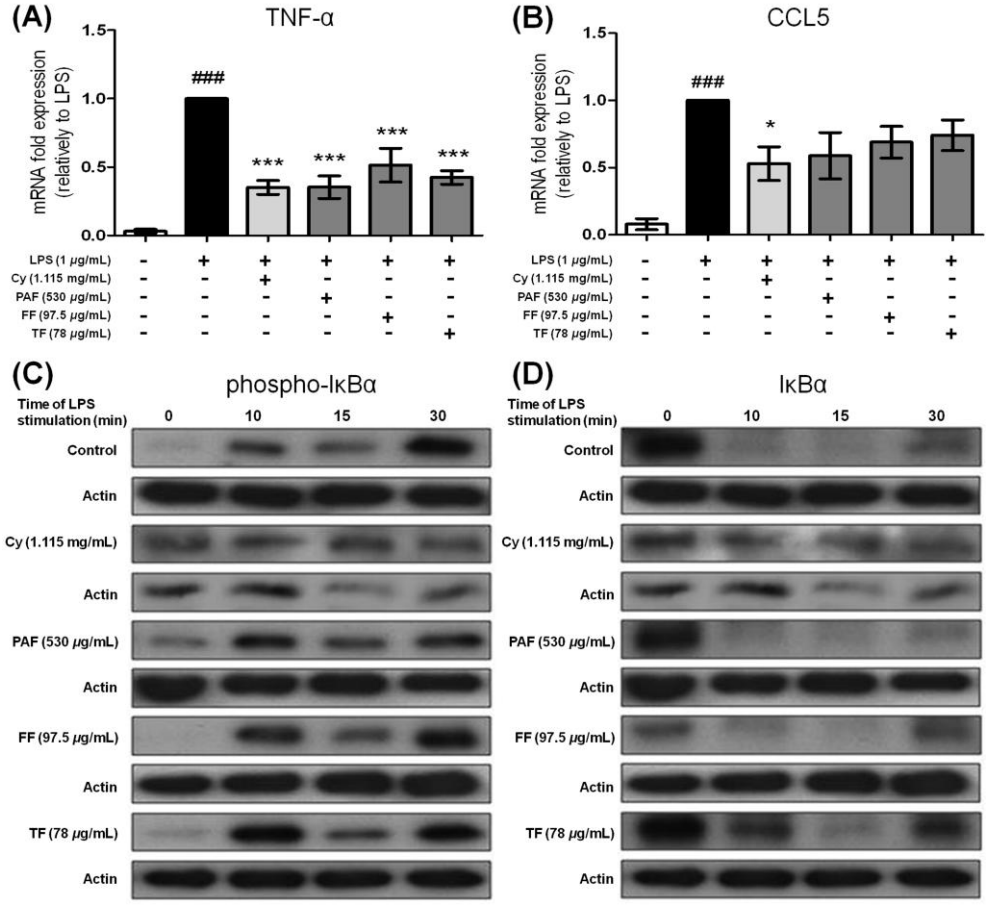

Figure 2.11. Cymbopogon citratus (Cy) and its polyphenol-rich fractions inhibited LPS-induced TNF-α (A) and CCL-5 (B) production as well as NF-kB activation (C and D), in human macrophages. In the graphics, the results were expressed as fold changes relatively to LPS and each value represents the mean±SEM 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-kB activation was analyzed by western blot using antibodies against phospho-IkBα (C) and total IkBα (D). Each blot shown is representative of 3 blots yielding similar results.

3.3. NF-kB activation

The expression of pro-inflammatory cytokines is mainly regulated by NF-kB pathway. So, the effect of Cy extract and its PFs on NF-kB activation was assessed by western blot and reporter assays. The pre-treatment of human macrophages with Cy extract, maintained the LPS-induced IkBα phosphorylation (Fig. 2.11C) and blocked the degradation of IkBα (Fig. 2.11D), suggesting the inhibition of NF-kB activation. Additionally, the inhibition of IkBα 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 IkBα (Fig. 2.12A) and the blockade of IkBα degradation induced by LPS (Fig. 2.12B). It was verified that none of the three fractions have a significant effect in LPS-induced IkBα phosphorylation, but PAF and FF inhibited the IkBα degradation (Fig. 2.12B). Interestingly, a standard of the main phenolic acid in Cy extract, the chlorogenic acid, maintained the phosphorylation levels of IkBα, 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. 2.12C and 2.12D), this result being consistent with a NF-kB inhibition by Cy. Additionally, all the PFs inhibited the transcriptional activity of NF-kB (Fig. 2.12E), thus suggesting their contribution to the Cy extract anti-inflammatory potential by inhibition of NF-kB activation.

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

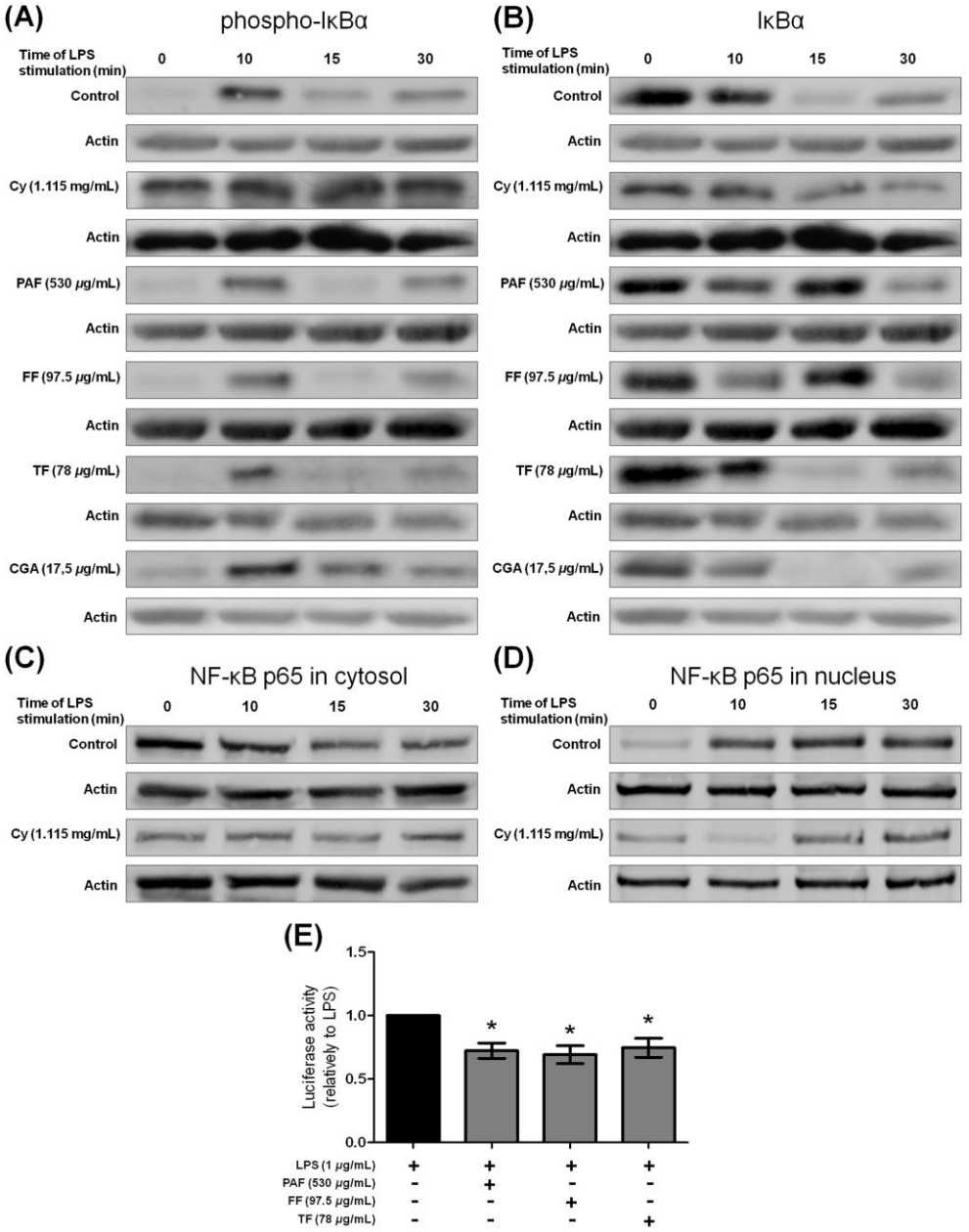

Figure 2.12. Cymbopogon citratus (Cy) and its PFs inhibited LPS-induced NF-kB activation, in murine macrophages. Total cell extracts were analyzed by western blot using antibodies against phospho-IkBα (A) and total IkBα (B). The migration of NF-kB p65 to the nucleus was analyzed using cytoplasmic (C) and nuclear (D) extracts. Each blot shown is representative of 3 blots yielding similar results. The NF-kB transcriptional activity was measured using a NF-kB-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).

3.4. Proteasome activity

The ubiquitin-proteasome system has a central role in the regulation of NF-kB as well as 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. 2.13), which is concordant with the blockade of IkBα degradation and maintenance of its phosphorylation status. Both phenolic acid- and tannin-rich fractions inhibited proteasome activity; in particular, PAF decreased the proteasome activity by 24.5±9.8%, suggesting a 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-kB activation and, consequently, cytokines expression.

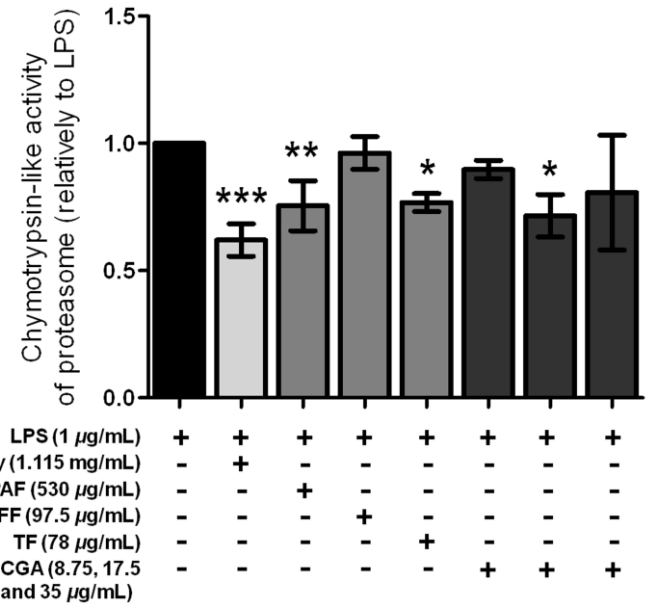

Figure 2.13. Cymbopogon citratus (Cy), phenolic acid-rich fraction (PAF) and chlorogenic acid (CGA) inhibited the chymotrypsin-like activity of murine macrophage proteasome. Proteasome activity was assessed using a fluorogenic peptide. The results were expressed as fold changes relatively to LPS and each value represents the mean±SEM from 3 independent experiments (*$p<0.05$ and **$p<0.01$, when compared to LPS).

3.5. Nitric oxide production

Given the inhibitory activity of CGA on NF-kB 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. 2.14), 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).

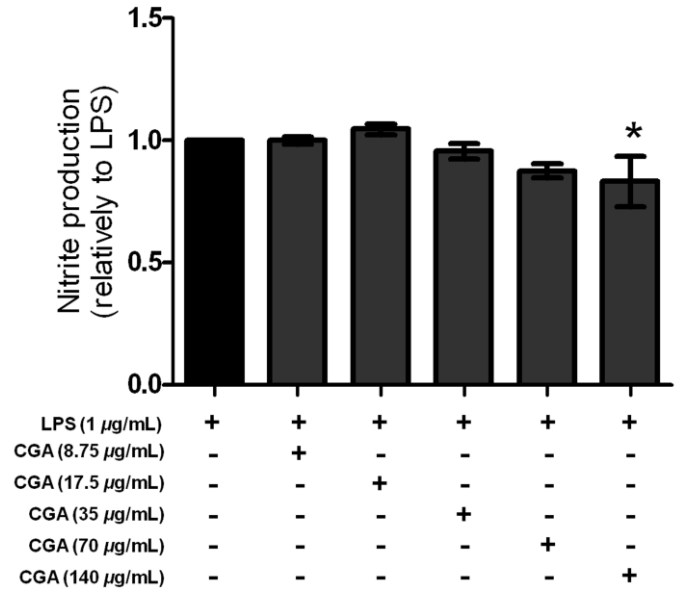

Figure 2.14. Chlorogenic acid (CGA) inhibited the LPS-induced nitrite production, in murine macrophages. The results were expressed as fold changes relatively to LPS and each value represents the mean ± SEM from 3 independent experiments (*$p<0.05$, when compared to LPS).

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-kB 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-kB pathway (Chen *et al.*, 2007). NF-kB 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-kB pathway inhibition. Consistent with this mechanism, we previously described the decrease in NF-kB-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-kB 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-kB and MAPK could be involved in the inhibition of LPS-induced TNF-α production by Cy extract. Supporting our data, Cy extract also

possesses *p*-coumaric derivatives that were previously described as inhibitors of NF-kB-dependent iNOS and COX-2 expression (Yen *et al.*, 2008).

The inhibition of ubiquitin-proteasome system, with consequent blockade of NF-kB pathway, reveals the anti-inflammatory potential of Cy extract. In fact, proteasome regulates protein degradation and homeostasis, having a crucial 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 μg/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. CGA was previously described as a proteasome inhibitor and anti-neoplasic agent (Cichocki *et al.*, 2010), as well as a NF-kB inhibitor (Shan *et al.*, 2009), which reinforces our results. Accordingly, our data demonstrated that CGA maintains the phosphorylation levels of IkBα, which evidences an inhibition of both proteasome activity and NF-kB activation. Since NF-kB 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-kB 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-kB pathway and cytokine expression. Additionally, phenolic compounds, in particular chlorogenic acid, were pointed as bioactive compounds, revealing the importance of *C. citratus* as source of new anti-inflammatory natural drugs.

C. Anti-inflammatory properties of *Cymbopogon citratus* flavonoids: a structure-activity relationship

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Abstract

Cymbopogon citratus (DC.) Stapf (Cy), Poaceae, commonly known as lemongrass, has been used in traditional medicine for the treatment of inflammatory-related diseases. Based on a previous study from our group demonstrating the contribution of a flavonoid-rich fraction to Cy anti-inflammatory activity in bio-guided assays, the aim of this work was to characterize flavonoids from Cy leaf infusion and to evaluate their anti-inflammatory activity.

O-, *C*- and *O,C*-glycosides of luteolin present in Cy were isolated and identified by proton nuclear magnetic resonance spectroscopy, namely luteolin 7-*O*-*β*-glucopyranoside, luteolin 6-*C*-*β*-glucopyranoside (isoorientin) and luteolin 2"-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B), being the last one identified for the first time in Cy. The cytotoxicity and the anti-inflammatory properties of the luteolin and its glycosides were evaluated in lipopolysaccharide-stimulated RAW 264.7 macrophages. Luteolin glycosides demonstrated less cytotoxicity than luteolin aglycone, which could be an advantage from a pharmaceutical point of view due to the high toxicity of the current available ant-inflammatory drugs. To evaluate the anti-inflammatory potential of the luteolin glycosides, the production of nitric oxide as well the expression of inducible nitric oxide synthase, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α were analyzed. The data demonstrated that glycosylation decreased the luteolin anti-inflammatory activity, the decrease being higher in the case of *C*-glycosylation than *O*-glycosylation. However, taking into account the bioavailability and metabolism of luteolin glycosides, their *in vivo* administration should be considered.

In conclusion, luteolin glycosides from *Cymbopogon citratus* are promising anti-inflammatory compounds, exhibiting greater toxicological safety than luteolin itself.

1. Introduction

Inflammation is a physiological protective process against harmful stimuli, such as infection from bacteria, viruses and other pathogens, and physical or chemical injury. In an initial stage, the activation of the immune cells leads to the release of several inflammatory mediators, such as nitric oxide (NO) and cytokines, namely interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α (Fortin *et al.*, 2010; Kopf *et al.*, 2010). This process persists only for a short period and is required for immune surveillance, optimal repair and regeneration after injury. However, when acute inflammation lasts for a longer period of time, chronic inflammation occurs, which is pointed out as a major mechanism in the pathogenesis of chronic illness, including diabetes, cardiovascular diseases, atherosclerosis, Alzheimer and cancer (Liu and Zeng, 2012; Osborn and Olefsky, 2012; Price *et al.*, 2012). Since the overproduction of pro-inflammatory mediators raises and maintains inflammation, compounds targeting their expression are good candidates for attenuating inflammatory diseases.

Cymbopogon citratus (DC.) Stapf (Cy), Poaceae, is a spontaneous perennial graminoid, commonly known as lemongrass. This plant is native from the southwest Asia and currently it grows around the world, mainly in tropical and subtropical countries. The essential oil from the plant leaves and their main phytochemical – citral, are used in the food, perfumery, soap, cosmetic, pharmaceutical and insecticide industries (Negrelle and Gomes, 2007). In traditional medicine, aqueous extracts of dried leaves are used for the treatment of several inflammation-based pathologies as well as other health problems (Shah *et al.*, 2011).

Flavonoids, characterized by a diphenylpropane structure (C6-C3-C6), are a large group of plant secondary metabolites commonly found in fruits, vegetables and certain beverages (Manach *et al.*, 2004). The chemical diversity, size, three-dimensional shape, and physical and biochemical properties of flavonoids, allow their interaction with multiple targets to influence biological activity in plants, animals and microorganisms. In fact, many therapeutic properties have been assigned to flavonoids, namely anti-inflammatory (Middleton *et al.*, 2000; Prasad *et al.*, 2010). We previously described that the main flavonoids of *Cymbopogon citratus* leaf infusion are luteolin glycosides (Figueirinha *et al.*, 2008). Numerous experimental data have demonstrated that luteolin have a wide range of biological activities including antioxidant, antimicrobial, anticancer, anti-allergic and anti-inflammatory (for extensive review see López-Lázaro, 2009), which highlighted the

valuable therapeutic potential of luteolin and luteolin derivatives, namely luteolin glycosides.

The present research aimed to further characterize the flavonoids from *Cymbopogon citratus* leaf infusion by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and to evaluate the anti-inflammatory activity of its isolated compounds using the *in vitro* model of lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Structure-activity relationships of luteolin glycosides were also disclosed.

2. Materials and methods

2.1. General experimental procedures

Dulbecco's Modified Eagle Medium, penicillin, streptomycin, LPS from *Escherichia coli* (serotype 026:B6) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide were obtained from Sigma–Aldrich Química (St Louis, MO, USA). Fetal bovine serum was from Gibco (Paisley, UK) and Trizol® reagent was from Invitrogen (Carlsbad, CA, USA). Luteolin, luteolin 7-*O*-*β*-glucopyranoside, luteolin 6-*C*-*β*-glucopyranoside and luteolin 8-*C*-*β*-glucopyranoside were from Extrasynthese (Genay, France). iScript™ select cDNA synthesis kit and SYBR-Green were purchased to BioRad (Hercules, CA, USA). Primers were from MWG Biotech (Ebersberg, Germany).

2.2. Plant material

Dry leaves of *Cymbopogon citratus* (DC.) 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).

2.3. Extraction and isolation of flavonoids

An essential oil-free infusion of *Cymbopogon citratus* was prepared and fractionated by column chromatography as previously described (Figueirinha *et al.*, 2008). All the fractionation process was monitored by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) for polyphenols. The flavonoid-rich fraction (yield of 4.4%, w/w, of Cy extract) was used as the starting point to isolate luteolin 7-*O*-*β*-

glucopyranoside, luteolin 6-*C*-*β*-glucopyranoside and luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) for structural characterization by ^{1}H NMR, and the luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl), commonly known as cassiaoccidentalin B (IUPAC: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-6-[(2S,5R)-5-hydroxy-6-methyl-4-oxo-3-[(2S,3S,5R)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]-chromen-4-one), was also isolated for the anti-inflammatory activity evaluation. Freeze-dried flavonoid-rich fraction was dissolved in methanol and applied on chromatography cellulose paper sheets (46x57cm) Whatman® 3MM (Maldstone, England), and eluted in a saturated chamber with 15% acetic acid. Isolated flavonoids were detected by UV (366 nm) observation. Each spot was removed from the paper by 50% methanol extraction and purified on polyamide. Structural elucidation of each isolated flavonoid was achieved by ^{1}H NMR.

2.4. ^{1}H NMR

^{1}H NMR spectra as well as 2D NMR data were obtained on a Varian VNMRS-600 NMR (^{1}H NMR at 600 MHz) spectrometer, at 25ºC and Methanol-d4 (CD_3OD) as solvent, with water signal suppression using pre-saturation technique. Chemical shifts were calculated through methyl sign of the solvent CD_3OD (δ_H 3.30 ppm). The 1D spectra were acquired in phase sensitive mode with spectral bandwidth of 5411.3 Hz (8.5 ppm to 0.5ppm) and relaxation time of 2 seconds. Each sample data were acquired using 8-16 accumulations with 16k points. Bi-dimensional spectra (2D) (gCOSY) and (TOCSY) were acquired in sensitive phase States-Haberkorn type with spectral bandwidth of 5411.3 Hz in both dimensions and a relaxation time of 2 seconds. TOCSY spectra were obtained using mixing times of 60 ms (3 relays) while for the gCOSY spectra was used 2K points data scans for each of 800 increments of t1 used. The accumulation number varied between 8 and 24 depending on the concentration of each sample. Data treatment of all spectra was realized using MestreNova software, v. 5.3.2-4936/2009.

2.5. Cell culture and experimental treatment

RAW 264.7 macrophages (ATCC number: TIB-71) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) non-inactivated fetal bovine serum, 100 *U*/mL penicillin and 100 *μ*g/mL streptomycin. The cells were cultured at 37°C in a humified atmosphere of 95% air and 5% CO_2.

For the experiments, cells were maintained in culture medium (control) or pre-incubated with the indicated concentrations of luteolin, luteolin 7-*O*-*β*-glucopyranoside,

luteolin 6-*C*-*β*-glucopyranoside (isoorientin), luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B) or luteolin 8-*C*-*β*-glucopyranoside (orientin). The luteolin derivatives concentrations were reported to luteolin content. Then, cells were activated with 1 μg/mL LPS from *Escherichia coli* (serotype 026:B6) for either 24h, to analyze cell viability and nitrite production, or for 8h to study cytokine expression evaluation.

2.6. Cell viability

Metabolically active cells were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric assay, as previously reported (Francisco *et al.*, 2011).

2.7. Nitrite production

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

2.8. iNOS and cytokine expression

After experimental treatment, total RNA was isolated from cells with Trizol® reagent. 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 iScript™ select cDNA synthesis kit (BioRad, Hercules, CA, USA), accordingly to manufacturer´s instructions, on C1000™ Thermal Cycler (BioRad, Hercules, CA, USA). The resulting cDNA of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1), iNOS, TNF-α, IL-1β and IL-6 were amplified by real-time RT-PCR, using the SYBR-Green assay to monitor the amplification reactions on a Bio-Rad My Cycler iQ5 (BioRad, Hercules, CA, USA). For that, specific primers were designed using Beacon Designer® Software v7.2 (Primier Biosoft International, Palo Alto, CA, USA) (Table 2.3). 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 HPRT1.

2.9. Statistical analysis

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.

Table 2.3. Oligonucleotide primer pairs used for real-time RT-PCR.

Gene name	Primer sequences (5′-3′)
HPRT1	F: GTTGAAGATATAATTGACACTG
	R: GGCATATCCAACAACAAAC
iNOS	F: GCTGTTAGAGACACTTCTGAG
	R: CACTTTGGTAGGATTTGACTTTG
TNF-α	F: CAAGGGACTAGCCAGGAG
	R: TGCCTCTTCTGCCAGTTC
IL-1β	F: ACCTGTCCTGTGTAATGAAAG
	R: GCTTGTGCTCTGCTTGTG
IL-6	F: TTCCATCCAGTTGCCTTC
	R: TTCTCATTTCCACGATTTCC

F: Forward sequence; R: Reverse sequence.

3. Results and discussion

3.1. Structural characterization of flavonoids by ^{1}H NMR

Flavonoids, namely the luteolin, are typically found in plants as glycosides (Manach 2004; López-Lazaro 2009), such as identified in *Cymbopogon citratus* (Figueirinha *et al.*, 2008). Besides the previous identification of luteolin *O*- and *C*-glycosides as the main Cy flavonoids, using high performance liquid chromatography, coupled to photodiode-array and electrospray ionization mass spectrometry detectors (HPLC-PDA-ESI/MS) (Figueirinha *et al.*, 2008), a complete structure elucidation of some Cy flavonoids was performed in this study. The proton nuclear magnetic resonance (^{1}H NMR) detects the interaction between the right frequency of radio waves and the hydrogen atom, which is determined by the hydrogen atom' s environment in the molecule and, therefore, allows the structure elucidation of the molecule. A full characterization of three Cy flavonoids by ^{1}H

NMR confirmed the presence of luteolin 7-*O*-*β*-glucopyranoside (L7OG) and luteolin 6-*C*-*β*-glucopyranoside (isoorientin; IsoO), which are representative of luteolin derivatives in Cy (Figueirinha *et al.*, 2008), and identified luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B; Cas B) for the first time in this plant. The structures of identified compounds are represented in Fig. 2.15 A-C and the ^{1}H NMR data are presented below.

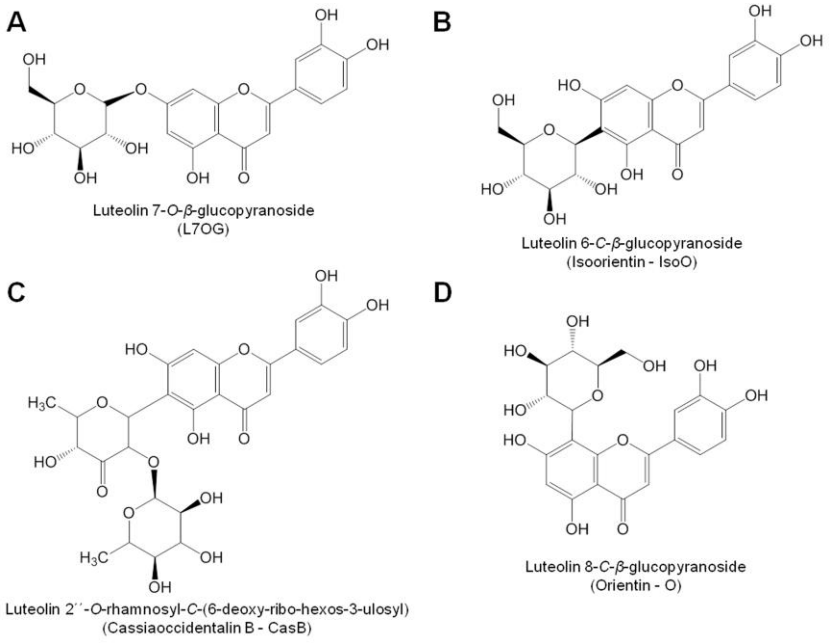

Figure 2.15. Chemical structures of luteolin glycosides from *Cymbopogon citratus* (DC.) Stapf leaves infusion characterized by ^{1}H NMR, namely luteolin 7-*O*-*β*-glucopyranoside (A), luteolin 6-*C*-*β*-glucopyranoside (B) and luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (C), and also luteolin 8-*C*-*β*-glucopyranoside (D).

3.1.1. Luteolin 7-O-β-glucopyranoside:

The presence of protons at carbons 6 and 8 in A ring, 3 in C ring and 2', 5' and 6' in B ring suggests that the aglycone may be the luteolin (Table 2.4). Accordingly, this compound presented a pseudomolecular ion at *m/z* 447 and a fragment at *m/z* 285 [(M-H)-162]$^{-}$, in MS^{2}, which corresponds to aglycone fragment (luteolin) by the loss of one hexose unit, UV spectra demonstrating a luteolin derivative with substitution at position 7 (Figueirinha *et al.*, 2008).

The ^{1}H NMR and 2D NMR spectra of this compound displayed an ABX coupling system existed through the signals at δ 7.40 (1H, dd, *J* = 8.3 Hz, H-6´), 7.39 (1H, d, *J* = 2.3, H-2´) and 6.90 (1H, d, *J* = 8.3 Hz, H-5´); the meta coupling between protons H-6 at δ 6.49

(1H, d, 2.2 Hz, H-6) and H-8 at δ 6.78 (1H, d, 2.2 Hz, H-8) and the singlet at δ 6.59 (1H, s, H-3). An anomeric proton at 5.06 (1H, d, *J* = 7.5 Hz, H-1") observed in the 1D spectrum exhibited a coupling constant resulting from the vicinal coupling with H-2´´ proton (Table 2.4). This behavior suggests the presence of a *β*-glucopyranoside residue, its chemical shift being similar to that described for 7-*O*-glucosides (Zhu *et al.*, 2010). The other protons of the sugar were identified using the correlation analysis obtained in the gCOSY spectra and compared with literature data, suggesting the existence of a *O*-*β*-glucopyranoside residue in this compound (Brito-Arias, 2007).

3.1.2. Luteolin 6-C-β-glucopyranoside (isoorientin):

The ^{1}H NMR spectrum of this compound displayed an ABX coupling system through the signals at δ 7.40 (2H, m, H-2', H-6'), 6.90 (1H, d, *J* = 8.1 Hz, H-5'). The singlet at 6.55 (1H, s, H-3) and 6.49 (1H, s, H-8) is consistent with location observed in flavones unsubstituted in those positions (Leong *et al.*, 2010). The existence of one proton in A ring was also confirmed by the gCOSY ^{1}H-^{1}H spectrum where only the coupling of the protons of B ring were visible.

The same spectrum also exhibited an anomeric proton at 4.90 (1H, d, *J* = 9.9 Hz, H-1") (Table 2.4). The large coupling constant (9.9 Hz) of the anomeric proton at δ 4.90 indicates a β configuration of the sugar (Rayyan *et al.*, 2005). The region between 3.8 and 4.2 exhibited five additional signals, which were related with the protons of a glucopyranoside moiety by the 2D NMR spectrum and comparison with literature data (Andersen and Markham, 2006).

3.1.3. Luteolin 2´´-O-rhamnosyl-C-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B)

The ^{1}H NMR spectrum of this compound displayed an ABX coupling system through the signals at δ 7.40 (2H, m, H-2', H-6'), 6.90 (1H, d, *J* = 8.3 Hz, H-5'). The same spectrum exhibited the singlet at 6.58 (1H, s) and 6.53 (1H, s) which, according to Hatano and co-workers (1999), can be attributed to H-3 and H-8, respectively (Table 2.4). The location of protons, identified by ^{1}H NMR, is consistent with a luteolin type structure for the aglycone. In agreement with these data, the MS fragmentation pattern and the UV spectra of this compound corroborated an *O*,*C*-diglycosylluteolin structure (Figueirinha *et al.*, 2008).

Due to the high overlay of the signals, the sugars protons assignment was made by selective ^{1}H-^{1}H TOCSY. The signals in the anomeric region at 4.80 and 4.62 ppm were

related to keto-osidic and rhamnose units respectively. The existence of two series of correlations, H-1´´-H-2´´ and H-4´´-H-5´´-H-6´´ in the ^{1}H-^{1}H TOCSY spectrum and the chemical shift exhibited by proton H-2´´ (5.24 ppm) are consistent with a ketone carbon at C-3 (Hatano *et al.*, 1999).

Table 2.4. ^{1}H NMR spectroscopic data (600 MHz, CD_3OD) for luteolin 6-*C*-*β*-glucopyranoside, luteolin 7-*O*-*β*-glucopyranoside and luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl).

POSITION	**Luteolin 7-*O*-*β*-glucopyranoside**	**Luteolin 6-*C*-*β*-glucopyranoside**	**Luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl)**
	δ_H (*J* in Hz)	**δ_H (*J* in Hz)**	**δ_H (*J* in Hz)**
3	6.59, s	6.55, s	6.58, s
6	6.49, d (2.2)	-	-
8	6.78, d (2.2)	6.49, s	6.53, s
2´	7.39, d (2.3)	7.40, m	7.40, m
5´	6.90, d (8.3)	6.90, d (8.1)	6.90, d (8.3)
6´	7.40, dd (8.3)	7.40, m	7.40, m
1´´	5.06, d (7.5)	4.90, d (9.9)	4.80, br
2´´	3.50, m (7.5, 9.5)	4.18, t	5.24, br
3´´	3.50, m (9.5, 9.0)	3.48, m	-
4´´	3.41, t (9.0, 9.7)	3.48, m	3.79, d (10)
5´´	3.54, m (9.7, 2.3, 5.8)	3.42, m	3.26, br m
6a´´	3.94, dd (2.3,12)	3.87, dd (12)	1.21, d (5.5)
6b´´	3.73, dd (5.8, 12)	3.74, dd (12)	-
1´´´	-	-	4.62
2´´´	-	-	3.72, m
3´´´	-	-	3.08, m
4´´´	-	-	2.92, m
5´´´	-	-	2.35, m
6´´´	-	-	0.64, s

3.2. Luteolin glycosylation decreases cytotoxicity

To determine the cytotoxic effect of luteolin (Lut), luteolin 7-*O*-*β*-glucopyranoside (L7OG), isoorientin (IsoO) and cassiaoccidentalin B (CasB) in LPS-stimulated RAW 264.7, the MTT assay was performed. To better characterize the structure-activity relationship, in particular if the position of *C*-glycosylation in the A ring affects the bioactivity, luteolin 8-*C*-*β*-glucopyranoside (orientin; O) was included in the study and its structure is represented in Fig. 2.15 D. As shown in Fig. 2.16, luteolin aglycone and luteolin glycosylated, showed no cell toxicity at 50 *μ*M. At the concentration of 100 *μ*M, luteolin aglycone had a strong cytotoxic effect, with a cell viability reduction of 98±1%, compared to LPS, while luteolin glycosides did not decrease cell viability, suggesting that luteolin glycosylation reduced luteolin aglycone cytoxicity. Furthermore, it was observed that luteolin 7-*O*-glucoside augmented MTT reduction to levels above LPS. Since the MTT method is based on the measurement of cellular metabolic activity via mitochondrial enzymes, L7OG effects in cell viability could be related to an increase in macrophage mitochondrial activity.

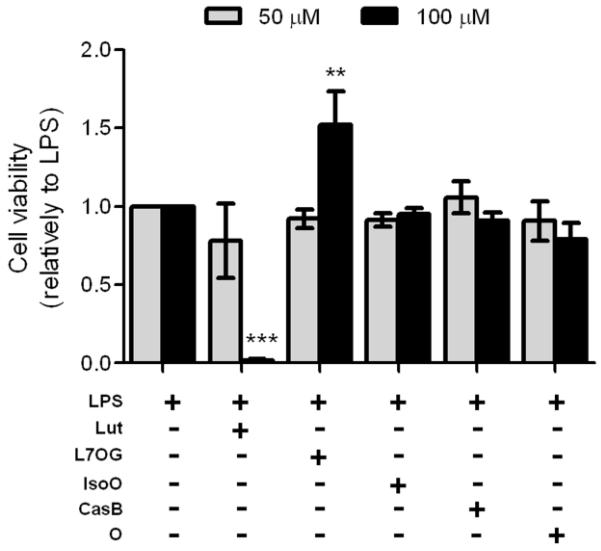

Figure 2.16. Cytotoxicity of luteolin and luteolin glycosides in LPS-stimulated RAW 264.7 macrophages. Cells were pre-incubated with either 50 or 100 *μ*M luteolin (Lut), luteolin 7-*O*-*β*-glucopyranoside (L7OG), isoorientin (IsoO), cassiaoccidentalin B (CasB) or orientin (O) for 1h and then stimulated with LPS for 24h. Cell viability was assessed using MTT assay and results expressed relatively to LPS-treated cells. Each value represents mean±SEM of at least 3 independent experiments (***$p < 0.001$, when compared to LPS).

Given the absence of cell toxicity detected for luteolin glycosides, these compounds should be explored as interesting alternatives to NSAID´s, which are largely used in clinic to treat inflammatory conditions but have gastrointestinal and renal adverse effects. Accordingly, isoorientin did not demonstrate any apparent acute toxicity as well as gastric damage when administered orally to rats (Küpeli *et al.*, 2004). Besides the lowest toxicity, glycosylation could provide other pharmaceutical advantages, namely a higher solubility and stability, and therefore a more desirable pharmacological effect. For instance, it is known that aglycones are not found in the plasma, or found in very small amounts, because of their hydrophobicity, so it is expected that a higher amount of luteolin reaches the target tissues if it is administered in glycoside form. Moreover, flavonoid glycosides are good metabolic substrates for intestinal sodium-dependent glucose cotransporters (SGLT), which enhances the *in vivo* activity of these molecules (Perez-Vizcaino *et al.*, 2012).

3.3. Luteolin glycosylation alters the anti-inflammatory activity

Previous analysis on flavonoid´s structure-activity relationships showed that a double bond at position C2-C3 of the C-ring with oxo function at position 4, together with the presence of four hydroxyl groups at positions 5, 7, 3' and 4' and the position of the B ring at 2, which is the structure of luteolin, seem to be required for the highest anti-inflammatory effect (Comalada *et al.*, 2006). In fact, the anti-inflammatory properties of luteolin have been extensively reported (for review see Seelinger *et al.*, 2008 and López-Lázaro, 2009); however, to the best of our knowledge, a comparative study on the effect of *C*- and *O*-glycosylation in luteolin anti-inflammatory activity was not performed yet. To analyze the effect of glycosylation in luteolin anti-inflammatory activity, the production of several inflammatory mediators, namely nitric oxide (NO) and cytokines were evaluated in macrophage cells, which have a crucial role during the inflammatory process by providing an immediate defense against foreign agents and have been previously used by us (Francisco *et al.*, 2011). The effect of luteolin aglycone and its glycosides on NO production triggered by LPS in macrophages was evaluated through measuring the nitrite accumulation in the culture medium by Griess reaction, as previously (Francisco *et al.*, 2011). In resting conditions, RAW 264.7 macrophages produced low levels of nitrites (0.11 ± 0.06, comparatively to LPS), which increased after cells activation with LPS for 24h (Fig. 2.17). Luteolin aglycone, at both concentrations, strongly suppressed nitrite production to levels similar to control. In contrast, isoorientin, cassiaoccidentalin B and orientin, all luteolin *C*-glycosides, showed no significant effect on nitrite production. On the other hand, at the

higher concentration of 100 μM, L7OG significantly inhibited the LPS-induced NO production by 44 ± 8% (relatively to LPS), thus indicating that L7OG has anti-inflammatory activity, although is lower than the observed with luteolin aglycone. Based on the effects in cell viability and NO production, the concentrations presenting bioactivity and without toxicity were selected for the further experiments and correspond to 50 μM for luteolin aglycone and 100 μM for luteolin glycosylated.

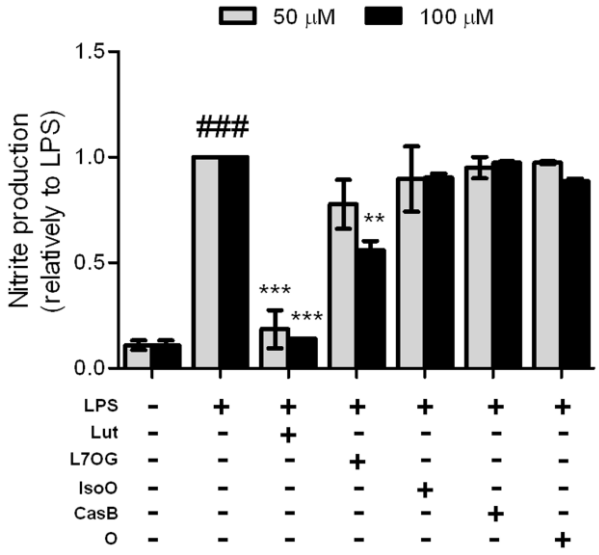

Figure 2.17. Effect of luteolin and luteolin glycosides on the LPS-induction of NO production. Cells were maintained in culture medium or pre-incubated with either 50 or 100 μM luteolin (Lut), luteolin 7-*O*-β-glucopyranoside (L7OG), isoorientin (IsoO), cassiaoccidentalin B (CasB) or orientin (O) for 1h before stimulation with LPS for 24h. Nitrite levels in the culture supernatants were evaluated by the Griess reaction and results expressed relatively to LPS-treated cells. Each value represents mean±SEM of at least 3 independent experiments ($^{###}p < 0.001$, when compared to control; $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared to LPS).

Since NO is synthesized from L-arginine by inducible NO synthase (iNOS), the effect on iNOS expression at the transcriptional level was investigated by real time RT-PCR. Similarly to the effect on nitrite production, a statistically significant inhibition of iNOS mRNA levels by luteolin and L7OG was observed (Fig. 2.18 A), and the effect was less prominent with L7OG than with luteolin aglycone. No inhibition was observed after treatment with isoorientin, cassiaoccidentalin B or orientin. These results indicate the correlation between the effects on NO production and iNOS expression, and demonstrate the anti-inflammatory effects of luteolin aglycone and L7OG, although at a lower extent.

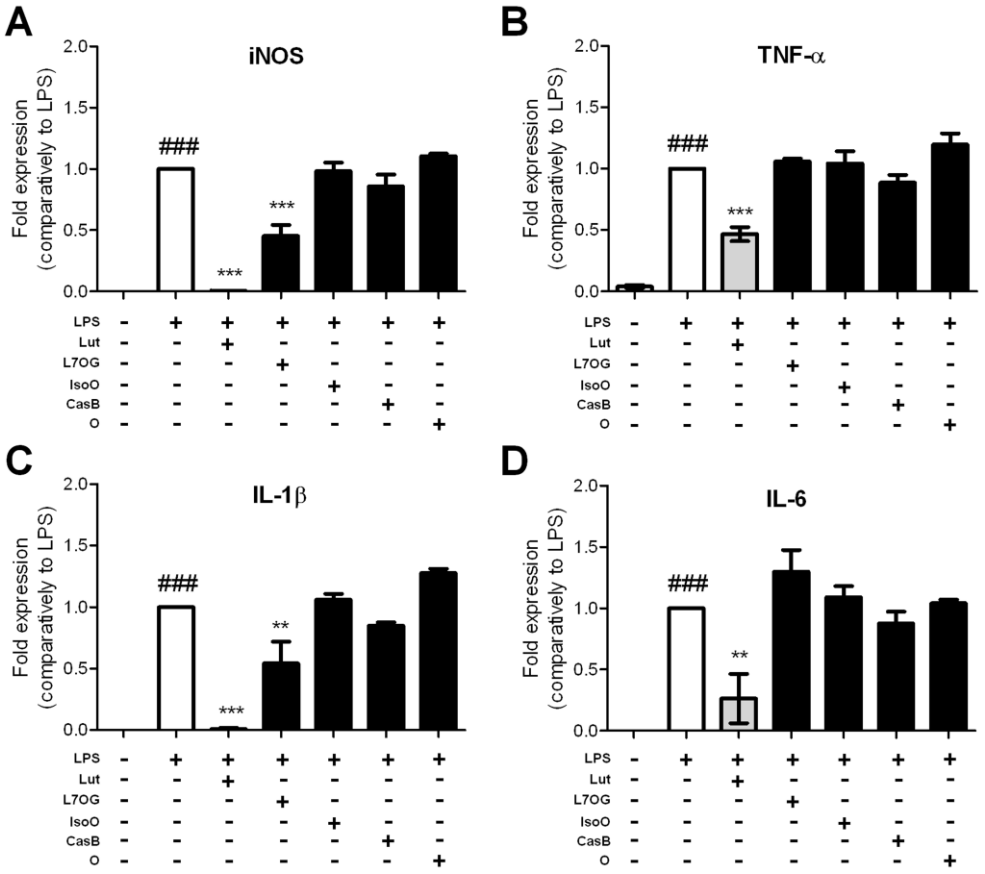

Figure 2.18. Effect of luteolin and luteolin glycosides on the LPS-induction of iNOS and cytokine expression. Cells were maintained in culture medium or pre-incubated with either 50 μM luteolin (Lut), 100 μM luteolin 7-*O*-β-glucopyranoside (L7OG), 100 μM isoorientin (IsoO), 100 μM cassiaoccidentalin B (CasB) or 100 μM orientin (O) for 1h before stimulation with LPS for 8h. Levels of iNOS (A), TNF-α (B), IL-1β (C) and IL-6 (D) mRNA was evaluated by real-time RT-PCR. The results were analyzed by the Pfaffl method and report gene expression as relative fold changes compared to LPS samples and normalized to the reference gene HPRT1. Each value represents the mean±SEM from 3 independent experiments performed in duplicate ($^{###}p < 0.001$, when compared to control; $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared to LPS).

Next, the effect of luteolin and luteolin derivatives on the expression of some cytokines, namely TNF-α, IL-1β and IL-6, was evaluated by real time RT-PCR. Luteolin aglycone significantly inhibited LPS-induced expression of TNF-α, IL-1β and IL-6 (Fig. 2.18). TNF-α and IL-6 expression was reduced by 53.3 ± 10.0% and 73.7 ± 34.7%, respectively, and IL-1β expression was completely abolished. Concerning to the effect of luteolin glycosylated, only L7OG modulated cytokine expression by reducing the LPS-induced IL-1β expression by 45.7 ± 30.4%, thus demonstrating the L7OG anti-inflammatory properties by inhibiting NO and IL-1β production. Interestingly, the inhibition of NO production and iNOS expression by luteolin 5-*O*-glucoside was recently

reported (Jung *et al.*, 2012), thus indicating that the position of *O*-glycosylation did not affect the anti-inflammatory activity.

All together, the results demonstrated that glycosylation decreased luteolin bioactivity and the decrease was higher in the case of *C*-glycosylation than *O*-glycosylation in the A ring. Accordingly, previous data evidenced that glycosylation of apigenin and luteolin completely suppress their anti-inflammatory activity because glycosylation reduced the flavone absorption into systemic circulation and decreased apigenin uptake by macrophages (Hostetler *et al.*, 2012). However, in that study only TNF-α production was addressed by the authors and our results proved that L7OG did not inhibited TNF-α production but had other anti-inflammatory properties like NO and IL-1β inhibition. Therefore, several pro-inflammatory mediators should be analyzed in order to conclude with accuracy about the anti-inflammatory potential of new compounds. It is known that NF-kB activation by LPS up-regulates the expression of iNOS, TNF-α, IL-6 and IL-1β in macrophages (Vitiello *et al.*, 2012). Since luteolin 7-*O*-*β*-glucopyranoside modulates LPS-mediated induction of iNOS and IL-1β but not TNF-α and IL-6, we could hypothesize that other intracellular signaling pathways than NF-kB, could be modulated by this flavonoid, in LPS-stimulated RAW 264.7 macrophages, such as activator protein-1 (AP-1), peroxisome proliferator-activated receptors (PPARs), interferon regulatory factors (IRFs), among others.

Isoorientin and cassiaoccidentalin B, both 6-*C*-glycosides, as well as orientin, a 8-*C*-glycoside, showed no anti-inflammatory activity in the *in vitro* studies, indicating that *C*-glycosylation hindered the luteolin-mediated anti-inflammatory activity, independently of the position of *C*-glycosylation in the A ring. However, isoorientin has been reported to have anti-inflammatory activity in a carrageenan-induced paw edema mice model (Küpeli *et al.*, 2004). In addition, luteolin 7-*O*-glucoside also showed anti-inflammatory activity *in vivo*, in a murine asthmatic model (Jin *et al.*, 2009). These apparent discrepancies might be related with the bioavailability and metabolism of luteolin glycosides. Similarly to what occurs to quercetin and to the proposed metabolism for polyphenols (Kawai, 2011; Perez-Vizcaino *et al.*, 2012), luteolin 7-*O*-glucoside might be first hydrolyzed to aglycone, probably by either intestinal bacteria or by glycosidases at the small intestine, and further glucoronated by phase II detoxifying enzymes, before being released into blood serum. This might explain why luteolin glycosides were not found in the plasma and luteolin aglycone is only found in a small amount, glucuronated derivatives being the most abundant form of luteolin in the plasma (Shimoi *et al.*, 1998). During inflammation,

increased tight-junctions permeability in endothelial cells occurs (Yan *et al.*, 2005), a phenomenon that could be responsible by the accumulation of glucuronated flavonoids in the inflammatory site (Kawai, 2011). Additionally, inflammatory cells, such as macrophages, release lysosomal enzymes, including *β*-glucuronidase, which catalyze flavonoid deconjugation and then, the flavonoid aglycone could be taken by the cells much more efficiently (Kawai, 2011; Hostetler *et al.*, 2012). Therefore, luteolin glycosides could demonstrate low activity *in vitro* but higher activity *in vivo* because, *in vivo*, both luteolin glycosides and luteolin aglycone are metabolized to glucuronides that are selectively activated in inflammatory sites.

4. Conclusions

Luteolin glycosides identified in *Cymbopogon citratus* by ^{1}H NMR, namely luteolin 6-*C*-*β*-glucopyranoside (isoorientin), luteolin 7-*O*-*β*-glucopyranoside and luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B) demonstrated decreased cytotoxicity in comparison to luteolin aglycone, suggesting that these compounds could be an interesting alternative to NSAIDs, known to have gastrointestinal adverse effects. Additionally, glycosylation could provide other pharmacokinetic advantages, namely a higher solubility and stability.

The data presented here evidenced that the anti-inflammatory activity of luteolin is dependent on its glycosylation degree, allowing the establishment of structure-activity relationships. Independently of the position of *C*-glycosylation in the A ring, it decreases luteolin anti-inflammatory activity more than *O*-glycosylation, which reveals the importance of the link between aglycone and the sugar moiety to the luteolin bioactivity. It has to be taken into account that *in vivo*, both luteolin aglycone and luteolin glycosides could be metabolized to glucuronides that should be recognized as food-derived pro-drugs that are selectively activated at inflammatory sites were the expression of *β*-glucuronidase is high.

In summary, luteolin glycosides isolated from *Cymbopogon citratus* are promising anti-inflammatory compounds with important pharmacological advantages, comparing to luteolin aglycone, and *in vivo* experiments should be considered on further works.

CHAPTER III

GENERAL DISCUSSION AND CONCLUSIONS

General discussion and conclusions

Inflammation is a protective process by which body fights infection or injury and initiates the healing process. However, sustained inflammatory state or chronic inflammation may lead to pathological conditions, such as cancer, rheumatoid arthritis, diabesity, cardiovascular and neurodegenerative diseases (Schmidt and Duncan, 2003; Porta *et al.*, 2009; Whitney *et al.*, 2009; Hunter and Doddi, 2010). Current anti-inflammatory drugs have several limitations such as lack of responsiveness, side effects, delivery problems and high costs of manufacture. Therefore, the work developed in this thesis aimed to find new anti-inflammatory compounds with selective pharmacology and presenting a safe toxicological profile, using bio-guided assays. For that, macrophages were chose as cell model due to its key role in providing an immediate defense against foreign agents and linking innate and adaptative immune responses (Murray and Wynn, 2011). Upon activation with an inflammatory stimulus, such as LPS, macrophages produce several pro-inflammatory mediators, including NO, eicosanoids, cytokines, like TNF-α, IL-1β and IL-6, and chemokines, like CCL5 (Murray and Wynn, 2011; Verschoor *et al.*, 2012). Therefore, inhibition of pro-inflammatory mediators' production is a useful strategy to screen new anti-inflammatory drugs. Additionally, the expression of inflammatory mediators is tightly regulated by several transcription factors and signaling pathways, such as NF-kB, MAPKs, PI3K/Akt and ubiquitin-proteasome system, which make them potential molecular targets for novel anti-inflammatory therapeutics. So, the screening for new anti-inflammatory compounds was performed in the *in vitro* model of LPS-stimulated macrophages and through evaluation of the inflammatory mediators´ production and modulation of intracellular signaling pathways. The *in vitro* approaches for early determination of chemicals' therapeutic properties have gained special interest in the last years not only because of the 3 R's (replacement, reduction and refinement) but also because they are cost and time effective. Furthermore, it is possible to determine the therapeutic profile of compounds early in the drug discovery process, and, therefore, this information could be used to guide further modifications of the chemicals in order to obtain favorable metabolic properties.

Plant extracts have been used for centuries in traditional medicine to alleviate inflammatory conditions and, therefore, phytochemicals are considered good candidates in the search for new anti-inflammatory compounds. Indeed, most of the drugs actually available are derived from natural products, having plant-derived materials a strong

contribution (Cragg and Newman, 2013). Among the plant-derived compounds, polyphenols are promising molecules due to their well-known therapeutic properties, namely antioxidant, cardio- and neuro-protective, anti-cancer and anti-inflammatory (Stevenson and Hurst, 2007; González-Gallego *et al.*, 2010); thus, the focus of this work was this class of phytochemicals.

Cymbopogon citratus (DC.) Stapf is a plant used in traditional medicine to treat inflammatory conditions, digestive disorders, diabetes, nervous disorders and fever, as well as other health problems (Negrelle and Gomes, 2007; Shah *et al.*, 2011). Although aqueous extracts are the most used in folk medicine, its pharmacological activity, in particular its anti-inflammatory properties, as well as the action mechanisms of Cy and its active compounds were poorly understood. The urgent need to discover new anti-inflammatory drugs and the lack of knowledge on Cy infusion, prompted us to disclose its anti-inflammatory properties and bioactive compounds. Our previous studies on chemical characterization of a lipid- and essential oil-free infusion from Cy leaves, using HPLC-PDA-ESI/MS/MS, identified polyphenols, namely phenolic acids (caffeic and *p*-coumaric acid derivatives), flavone glycosides (apigenin and luteolin derivatives) and condensed tannins as the main phenolic compounds (Figueirinha *et al.*, 2008). Additionally, the high antioxidant activity of Cy infusion, as well as its anti-inflammatory properties in LPS-stimulated dendritic cells, were strongly correlated with the high polyphenolic content (Figueirinha *et al.*, 2008; Figueirinha *et al.*, 2010). To further understand the anti-inflammatory properties and action mechanisms of Cy infusion and the contribution of its polyphenols, the focus of this thesis was to evaluate their bioactivity in LPS-stimulated macrophages. The main results obtained are pointed out below and summarized in Fig. 3.1:

- The data demonstrated the strong anti-inflammatory properties of Cy leaves infusion through inhibition of NO and PGE_2 production, in LPS-stimulated murine macrophages, as well TNF-α and CCL-5 expression, in human macrophages stimulated with LPS.
- The action mechanism involved in Cy activity was also disclosed. It was verified, for the first time, that proteasome activity was inhibited by Cy and, consequently, NF-kB activation and expression of pro-inflammatory mediators, such as NO, TNF-α and CCL5, were also reduced. Furthermore, Cy inhibited the activation of p38 MAPK and JNK 1/2 signaling pathways, which are involved in Cy anti-inflammatory properties.

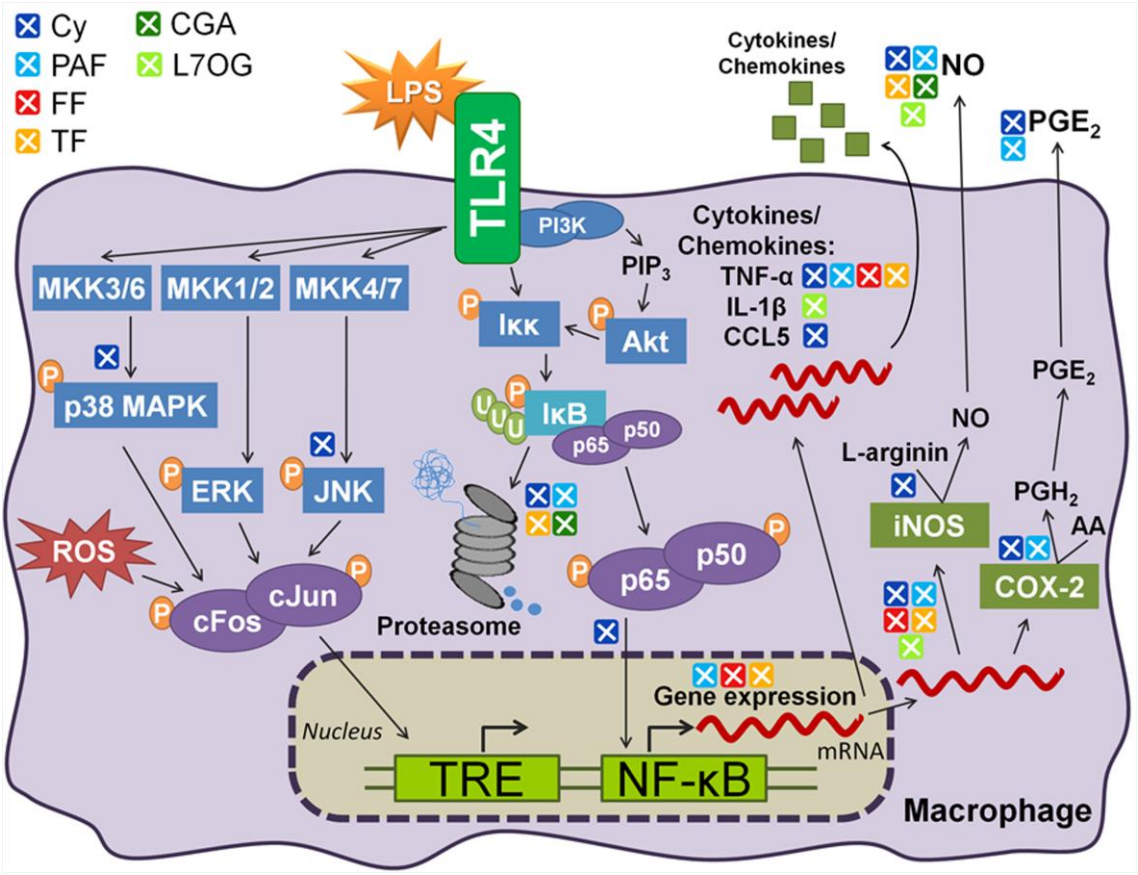

Figure 3.1. Anti-inflammatory action mechanisms of *Cymbopogon citratus* extract (Cy) and contribution of its polyphenol-rich fractions, namely phenolic acids (PAF), flavonoids (FF) and tannins (TF), as well as chlorogenic acid (CGA) and luteolin 7-*O*-*β*-glucopyranoside (L7OG).

▪ The polyphenols from Cy were partially responsible by its activity. Phenolic acids and tannins contributed to Cy anti-inflammatory properties through inhibition of LPS-induced TNF-α and iNOS expression, NO production and NF-kB activation, while flavonoids inhibited LPS-induced TNF-α and iNOS expression, as well as NF-kB activation.

▪ Chlorogenic acid was identified as the major compound of Cy extract using HPLC/PDA/ESI-MS^n. This phenolic acid was pointed out as bioactive compound, being the main responsible for proteasome inhibition and, consequently, inhibition of NF-kB activation and production of the pro-inflammatory mediator NO.

▪ Luteolin glycosides, from the flavonoid-rich fraction of Cy, were identified by ^{1}H NMR, namely luteolin 6-*C*-*β*-glucopyranoside (isoorientin) and luteolin 7-*O*-*β*-glucopyranoside, which are representative of luteolin derivatives in Cy, and luteolin 2´´-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B), identified for the first time in this plant. The data demonstrated that luteolin glycosides present lower toxicity

than luteolin aglycone, and evidenced the effect of glycosylation on the anti-inflammatory activity of luteolin, allowing the establishment of a structure-activity relationship. The decrease of luteolin anti-inflammatory activity achieved by *C*-glycosylation was more pronounced than *O*-glycosylation in the A ring, which reveals the importance of the link between aglycone and the sugar moiety to the luteolin bioactivity.

The data were obtained using a dynamic approach that conjugates chemical characterization, through HPLC/PDA, mass spectrometry and ^{1}H NMR, with the assessment of bioactivity, in particular anti-inflammatory properties, through measuring of NO and PGE_2 production, cytokine and chemokine expression, and also modulation of inflammation-related signaling pathways: *in vitro* bio-guided assays (Fig. 3.2).

The starting material - Cy leaves infusion - strongly inhibited the LPS-induced NO production, in murine macrophages, being the effect comparable to dexamethasone, a commercial anti-inflammatory drug. In general, the decrease of NO production results from a NO-scavenging capacity or modulation of iNOS expression and/or activity. Data showed that Cy did not have NO scavenging activity and reduced NO production at a bigger extend than iNOS expression, supporting an inhibition of NO production through modulation of both iNOS expression and activity. Additionally, an inhibition of TNF-α and CCL5 expression by Cy was also verified, in human macrophages. Corroborating these data, *in vivo* experiments, already performed in our laboratory, demonstrated the anti-inflammatory activity of Cy infusion using an acute model of inflammation, which supports the use of *in vitro* bio-guided assays to discover new anti-inflammatory phytochemicals.

Investigating the signaling pathways involved in Cy activity, we found an inhibition of the same pathways that are involved in NO production, namely NF-kB, p38 MAPK and JNK 1/2, indicating the contribution of these pathways to Cy anti-inflammatory properties. Since NF-kB is the master regulator of innate immunity (Geller and Billiar, 1998) and pointed out as a good target in the development of new anti-inflammatory drugs (Manna *et al.*, 2000), its inhibition was further investigated. It was demonstrated, for the first time, that Cy inhibited ubiquitin-proteasome system and, consequently, NF-kB pathway. Since the available proteasome inhibitors, like bortezomib, possess toxic effects (Gräwert and Groll, 2012), this interesting result points Cy and its compounds as a very attractive source of potent, but less toxic, proteasome inhibitors. Additionally, ubiquitin-proteasome system is a pivotal complex not only in inflammation but also in cancer development (DiDonato *et al.*, 2012), which encourages further investigation on Cy anti-neoplasic activity.

The production of pro-inflammatory mediator PGE_2 is also inhibited by Cy infusion but no effect was observed on COX-2 expression, the rate-limiting enzyme, indicating a modulation of COX-2 activity at concentrations presenting a safe toxicological profile to macrophages. The current treatment of inflammation is mainly based on NSAIDs that act through inhibition of COX, which reinforces the use of Cy as anti-inflammatory. Although inhibition of COX-2 activity has been associated with adverse renal and cardiotoxic effects (Harirforoosh and Jamali, 2009; Ritter *et al.*, 2009), in our previous studies, no histological changes were verified neither in the kidney nor in the liver using an *in vivo* acute inflammation model (unpublished data). However, it will be important to consider the effects of Cy long-term administration and its cardiotoxicy in future works.

As described above, Cy has a high content in polyphenolic compounds (Figueirinha *et al.*, 2008), that are secondary metabolites with described healthy effects (Stevenson and Hurst, 2007; González-Gallego *et al.*, 2010). To evaluate the contribution of polyphenols, Cy was fractionated by column chromatography providing three major fractions: PAF (yield of 23.8%, w/w, of Cy extract), FF (yield of 4.4%, w/w, of Cy extract) and TF (yield of 3.5%, w/w, of Cy extract). Therefore, the main compounds found in Cy were phenolic acids, followed by flavonoids and tannins. The aim of this study was to investigate the contribution of each fraction to the Cy activity and, therefore, the concentrations used were based on their ratios in the Cy extract after fractionation and without cytotoxicity. To evaluate the potency of each fraction, a study using the same concentrations of all the fractions should be performed. The results supported a strong contribution of polyphenols to the anti-inflammatory activity of Cy. In particular, phenolic acids and tannins decreased LPS-induced NO production in murine macrophages, through inhibition of iNOS expression and activity, and TNF-α expression in human macrophages, such as Cy extract. Flavonoids decreased TNF-α and iNOS expression but not NO production, indicating a strong activation of iNOS activity, probably through modulation of L-arginine bioavailability, the rate-limiting iNOS substrate, as previously described (Mori and Gotoh, 2000). All the fractions slightly inhibited CCL5 expression, but none with statistic significance. The inhibitory effects of each fraction are lower than the whole extract, which indicates a synergistic action between the diferent classes of polyphenols and/or a contribution of other compounds to Cy activity.

The PGE_2 levels, induced by LPS in murine macrophages, were decreased by PAF through inhibition of COX-2 activity, being the effect comparable to the Cy extract and thus, indicating the strong contribution of PAF to its activity. In contrast, flavonoids and

tannins did not inhibit PGE_2 production or COX-2 expression, suggesting that FF and TF could be used as anti-inflammatory agents avoiding the secondary effects associated with COX-2 inhibition.

Analysing the action mechanism of PFs, we verified that none of the fractions modulated the LPS-induced activation of MAPKs or Akt and so, other compounds contributed to the inhibition of p38 MAPK and JNK by Cy. In turn, all the fractions inhibited NF-kB activation at transcriptional level, having PAF and TF proteasome inhibitory action. These data support the anti-inflammatory properties of PFs through inhibition of NF-kB activation and, consequently, production of inflammatory mediators, namely NO production by PAF and TF, and TNF-α expression by PAF, FF and TF.

Cy alone (without LPS as inflammatory stimulus), but not polyphenol-rich fractions, demonstrated pro-inflammatory activity by increasing NO and PGE_2 production, iNOS and COX-2 expression, as well as MAPKs, Akt and NF-kB activation. The pro-inflammatory properties of Cy could be interesting to further explore as activators of the immune system and thus protecting the body against injury. In fact, other plants with immunostimulant properties have been used in the treatment of autoimmune diseases, allergic conditions, cancer, viral infections, etc. (Kumar *et al.*, 2011). However, a possible endotoxin contamination of Cy infusion must be discarded before concluding about its immunostimulant properties. For that, the Limulus amebocyte lysate test was conducted but no conclusion about endotoxin contamination could be performed because Cy infusion interferes with this assay. Thus, an endotoxin removal column was used and Cy bioactivity was tested before and after using it. Since Cy induced the NO production after passing it through the endotoxin removal system (see attach, Fig. I), we can conclude that Cy pro-inflammatory action is an intrinsic property of the extract not related to the polyphenolic content. Thus, non-phenolic fractions obtained during Cy fractionation were tested, but none of them showed pro-inflammatory activity (see attach, Fig. II). Additionally, Burana-Osot and co-workers (2010) have described the immunostimulating activity of essential oil-free decoction of Cy leaves and a correlation with water-soluble polysaccharides was achieved. Therefore, an evaluation of sugars content on Cy infusion was performed, revealing that 20% of Cy are uronic acid (3,78%) and neutral sugars, namely glucose (11,93%), galactose (1,73%), mannose (1,04%), rhamnose (0,70%), arabinose (0,58%) and xylose (0,19%) (see attach, Fig. III). The type of sugars founded as well its percentage let us to think that they are associated with other molecules, like flavonoids, and therefore are not responsible by pro-inflammatory properties of Cy. The immunostimulant properties of

plants are an issue that should be considered very carefully (Gertsch *et al.*, 2011) and therefore, further studies are needed. However, it is important to note that, in LPS-stimulated macrophages, the anti-inflammatory properties of Cy overlay its pro-inflammatory activity and that PFs did not show stimulatory effects, which supports further studies on PFs in the context of anti-inflammatory action of Cy.

Phenolic acids were the main polyphenols in Cy extract and were partially responsible by its anti-inflammatory properties through inhibition of NO, PGE_2 and TNF-α production, being the ubiquitin-proteasome system and, consequently, NF-kB pathway involved. To disclose the Cy bioactive compounds, the activity of the main phenolic acid from Cy, identified by HPLC/PDA/ESI-MS^n as chlorogenic acid, was investigated. Similarly to PFs, the used concentrations of CGA were based on its concentration in PAF (3.33%) and PAF ratio in the Cy extract (23.8%). The results demonstrated that CGA strongly inhibited proteasome activity and consequently NF-kB, similarly to Cy, which points CGA as bioactive compound. Reinforcing our results, CGA was previously described as proteasome inhibitor and anti-neoplasic agent (Cichocki *et al.*, 2010), as well as NF-kB inhibitor (Shan *et al.*, 2009). Besides its effect on proteasome activity, CGA only inhibited NO production at higher concentrations, indicating that other compounds have an higher contribution to the Cy anti-inflammatory activity or synergistic effects could occur. Therefore, these data points CGA as bioactive compound of Cy and encourages further research on CGA as inhibitor of ubiquitin-proteasome system and consequently as a therapeutical agent in diseases associated with proteasome deregulation, such as cancer.

Previous characterization of FF revealed luteolin glycosides as the main compounds of this fraction (Figueirinha *et al.*, 2008). Although the bioactivity of this fraction is lower, comparatively to PAF and TF, the numerous experimental data demonstrating the biological activity of luteolin, including anti-inflammatory, prompted us to further characterize the Cy flavonoids and to evaluate the anti-inflammatory activity of isolated compounds. *O*-, *C*- and *O*,*C*-glycosides of luteolin were isolated and identified by ^{1}H NMR, namely luteolin 7-*O*-glucoside, isoorientin and cassiaoccidentalin B, being the last one identified for the first time in Cy. The data evidenced a structure-activity relationship; glycosilation of luteolin in the A ring decreased the cytotoxicity of luteolin aglycone, suggesting that these compounds could be an interesting alternative to NSAIDs, with known adverse effects. Analysing the anti-inflammatory properties of luteolin glycosides, it was demonstrated that only lutelin 7-*O*-glucoside possess bioactivity by inhibition of NO production, through iNOS expression, and IL-1β expression. This indicates that C-

glycosylation hindered luteolin anti-inflammatory activity more than *O*-glycosylation in the A ring and reveals the importance of the link nature between aglycone and the sugar moiety. To evaluate the importance of the position of *C*-glycosylation to the bioactivity, orientin was also included in the study. The results showed that the reduced anti-inflammatory activity of luteolin glycosides is independent of the position of *C*-glycosylation in the A ring. Even though our *in vitro* data, the anti-inflammatory properties of isoorientin and luteolin 7-*O*-glucoside has been previously reported *in vivo* (Küpeli *et al.*, 2004; Jin *et al.*, 2009). This problably occurs as a result of *in vivo* metabolization of luteolin glycosides to glucuronides (Kawai, 2011; Pérez-Vizcaíno *et al.*, 2012) and selective activation in inflammatory sites (Kawai, 2011; Hostetler *et al.*, 2012). Therefore, luteolin glycosides from Cy are promissing anti-inflammatory compounds and further work on luteolin glycosides metabolization and i*n vivo* bioactivity should be considered.

This work evidenced the contribution of phenolic acids, namely chlorogenic acid, and flavonoids, in particular luteolin glycosides, to the anti-inflammatory activity of Cy infusion. Additionally, it was demonstrated that tannins also contributed to the Cy biological activity and it will be important to better characterize the chemical nature of this fraction and the action mechanism responsible for its activity.

In conclusion, this work supports the use of *Cymbopogon citratus* (DC.) Stapf leaves infusion in traditional medicine, reveals Cy as source of new anti-inflammatory natural drugs and discloses its mechanisms of action. Additionally, chlorogenic acid and luteolin glycosides were pointed as bioactive compounds, using *in vitro* bio-guided assays (Fig. 3.2). Therefore, the data strongly supports and encourages further work on Cy extract and its polyphenols for the treatment of inflammation-associated conditions such as cancer, rheumatoid arthritis, diabesity, cardiovascular and neurodegenerative diseases. Furthermore, the identification of bioactive compounds encouraged the development of a pharmaceutical formulation, which is currently ongoing in our laboratory. In particular, luteolin glycosides are promising candidates due to its low toxicity, comparatively to luteolin aglycone, and pharmacological advantages, namely higher hydrosolubility and stability.

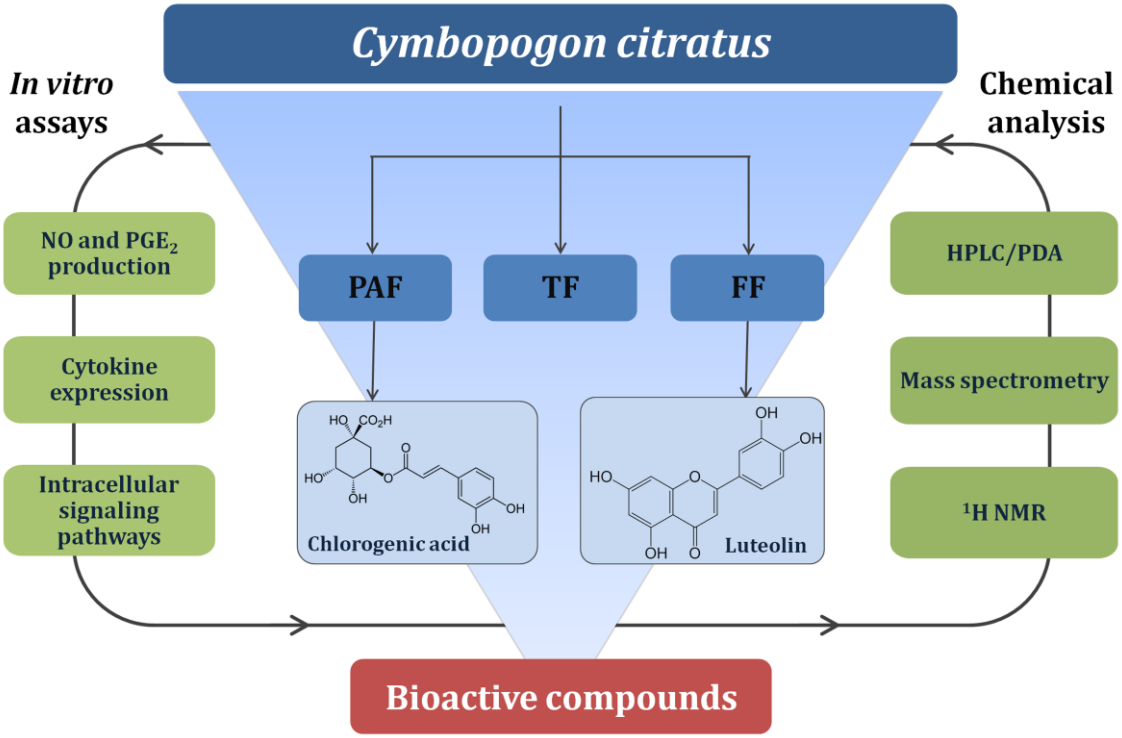

Figure 3.2. Schematic representation of *in vitro* bio-guided assays that allow the identification of bioactive compounds from *Cymbopogon citratus* (DC.) Stapf leaves infusion, namely chlorogenic acid and luteolin glycosides.

CHAPTER IV

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ATTACH

Cymbopogon citratus leaves infusion induced NO production after endotoxin removal

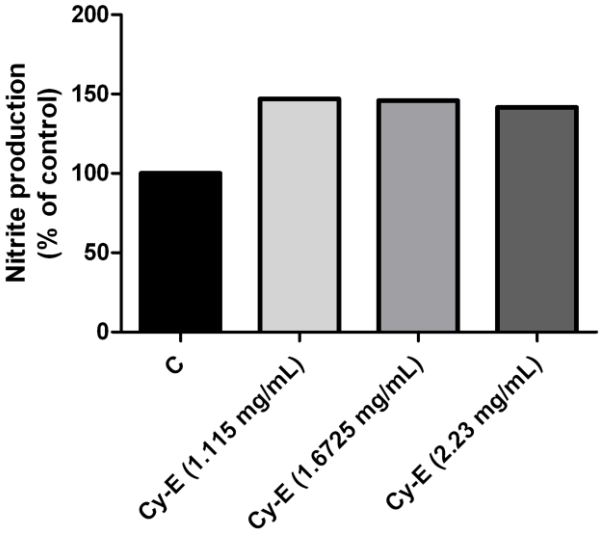

Figure I. Pro-inflammatory activity of *Cymbopogon citratus* (DC.) Stapf leaves infusion (Cy). An endotoxin removing system (Hycult Biotechnology b.v., Uden, The Netherlands) was used, to remove a possible endotoxin contamination of Cy extract, accordingly to the manufacturer´s instructions. The indicated concentrations of endotoxin-free Cy extract (Cy-E) were added to RAW 264.7 murine macrophages for 24h. Then, the nitrite levels in the culture supernatants were measured by Griess reaction, as previously described (Francisco *et al.*, 2011). One experiment was performed and the results were expressed as percentage of control.

Non-polyphenolic fractions of *Cymbopogon citratus* leaves infusion have no pro-inflammatory activity

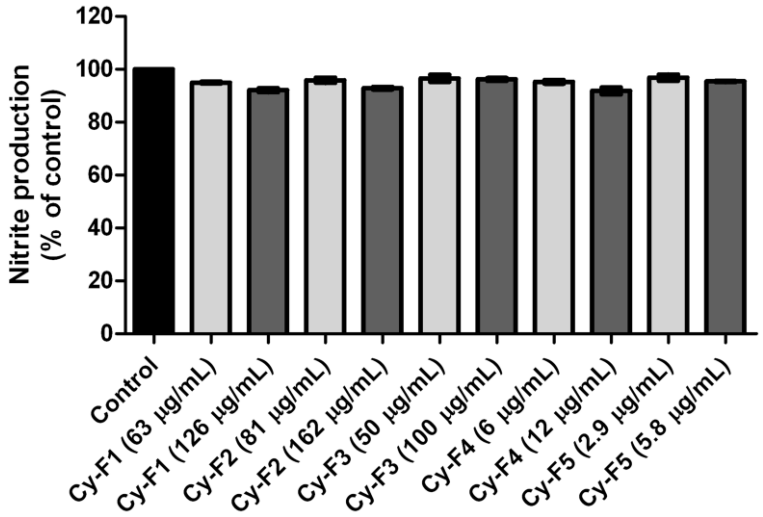

Figure II. Effect of non-polyphenolic fractions of *Cymbopogon citratus* (DC.) Stapf leaves infusion (Cy) in its pro-inflammatory activity. Cy was fractionated as previously (Figueirinha *et al.*, 2008) yielding non-polyphenolic fractions (Cy-F1 to Cy-F5). RAW 264.7 murine macrophages were incubated with the indicated concentrations of Cy fractions for 24h. The concentrations used were based in their ratio on Cy extract. Then, the nitrite levels in the culture supernatants were measured by Griess reaction, as previously described (Francisco *et al.*, 2011). Each value represents the mean±SEM from 3 independent experiments.

Sugars quantification in *Cymbopogon citratus* leaves infusion

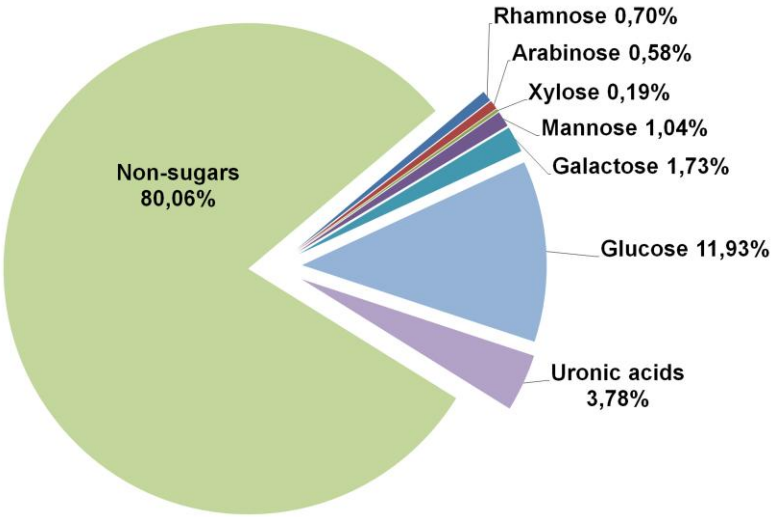

Figure III. Sugars quantification in *Cymbopogon citratus* (DC.) Stapf leaves infusion. Neutral sugars and uronic acid were quantified by chemical and spectroscopic analysis as previously described (Coimbra *et al.*, 1996)[1]

[1]Coimbra, M.A., Delgadillo, I., Waldron, K.W., Selvendran, R.R., 1996. Isolation and analysis of cell wall polymers from olive pulp. Modern Methods of Plant Analysis 17, 19-44