Gustavo Franco Ferreira da Costa

Cymbopogon citratus and its polyphenols as potential phytotherapeutic products: an *in vivo* approach

Doctoral Thesis in Pharmacy, specialization in Pharmacognosy and Phytochemistry, supervised by Professor Maria Teresa Batista and by Professor Isabel Vitória Figueiredo and presented to the Faculty of Pharmacy of the University of Coimbra.

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A thesis presented to the Faculty of Pharmacy of the University of Coimbra in fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacy (specialty in Pharmacognosy and Phytochemistry).

Front cover:

Lemongrass, acrylic on canvas

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(Pr 9, 1-5)

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Publications and communications

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

Chapters in international scientific books with referees:

Francisco V, Costa G, Neves BM, Cruz MT, Batista MT. **2014**. Anti-inflammatory Activity of Polyphenols on Dendritic Cells; Watson: Inflammation and Polyphenols; Polyphenols in Human Health and Disease; Academic Press; Elsevier. 28: 373-393. ISBN: 978-0-1239-8456-2.

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Abstract

Cymbopogon citratus (DC). Stapf (Poaceae), commonly known as lemongrass, is a tropical perennial shrub originated from the Southeast Asia. This plant is reported to possess antifungal, insecticidal, anti-diabetic, anti-septic, anti-mutagenic, anti-carcinogenic activities as well as anti-inflammatory. In fact, aqueous extracts of dried leaves are used all over the year in folk medicine for the treatment of peptic ulcers and inflammatory conditions. Recently, some phenolic compounds, such as luteolin and apigenin glycosides and condensed tannins, were identified and related to both antioxidant and anti-inflammatory properties. The purposes of this work were to i) validate an analytical method for quantification of phenolic compounds of *C. citratus*; ii) study the influence of harvest time and plant quality on the phenolic composition and antioxidant activity; iii) characterize the tannins; iv) validate the traditional uses of lemongrass infusion as anti-inflammatory *in vivo*; v) obtain a topical formulation to evaluate the phenolic compounds permeation and their anti-inflammatory activity; vi) trace the pharmacokinetic profile of the main phenolic compounds in rats.

Three different extracts: infusion (CcI), 50% aqueous ethanol (CcM50%) and ethanol (CcM100%) extracts were prepared and a simple and efficient RP-HPLC-PDA method was successfully validated for simultaneous identification and quantification of phenolic acids and flavonoids. Infusions were also obtained from different harvest dates (April, June, July, August and September) and quality grades (High, Medium and Low). It was verified that the content on polyphenols and the antioxidant capacity of CcI is strongly related with the quality of the plant. The total phenols assay showed a substantial loss from August to September. It was possible to find out the best month to harvest the plant to get the most of each phenolic group: April and June for hydroxycinnamic acids; June and September for flavonoids; June, July and August for tannins. Regardless the group of phenolic compound addressed, its content was always inversely proportional to the degree of leaves ageing. For all tested oxidant species, the high-quality samples exhibited the best antioxidant results.

CcI was fractionated by column chromatography and polyphenol-rich fractions, namely phenolic acids (CcPA), flavonoids (CcF) and tannins (CcT) were obtained. CcT was characterized by HPLC-PDA-ESI/MS^n, revealing the presence of proanthocyanidin hetero-dimers, along with some common procyanidin dimers. These hetero-dimeric flavan structures have been described for the first time in lemongrass and consist of apigeniflavan or luteoliflavan units linked to a flavanone, either naringenin or eriodictyol, and may occur as aglycone or glycosylated forms.

For the *in vivo* assays, CcI, CcF and CcT were tested. CcI administered before and after ethanol stimulus, significantly reduced the incidence and severity of gastric lesions and, consequently, the ulcer index, corroborating the traditional medicinal use of this plant to ameliorate gastritis and/or peptic ulcers symptoms. On the other hand, CcI, CcF and CcT were orally administered to rats, in order to evaluate the anti-inflammatory effect at the carrageenan-induced paw edema assay. The observed effect by CcI (68.24 mg/kg), 82.30% of edema inhibition, was very similar to that obtained by the reference NSAID used (diclofenac, 10 mg/kg), 84.00%. On the other hand, flavonoid (7.42 mg/kg) and the tannin-rich (5.96 mg/kg) fractions significantly contributed for the anti-inflammatory activity on the edema volume (59 and 61%, respectively).

The topical anti-inflammatory activity of CcI was also addressed. The results suggest that flavonoids, mainly, luteolin 7-*O*-neohesperidoside, cassiaoccidentalin B, carlinoside and cynaroside, may contribute to the topical anti-inflammatory effect. CcF (0.6%), CcT (0.3%) and CcF+CcT (0.66%+0.34%) topical formulations were also tested, and the results obtained suggest that tannins and flavonoids also possess a significant activity and that a synergistic mechanism of action may occur. In fact, edema inhibitions of 43%, 47% and 59% were respectively verified, being CcF+CcT effect very close to that of 1% diclofenac (65.9%).

Pharmacokinetic analysis was performed in plasma, liver and kidney and showed that the compounds present in CcI are not detected *in vivo* after a single-dose oral administration. In contrary, the metabolites, luteolin 7-*O*-glucuronide

and luteolin 3'-*O*-sulfate, present at the highest bioavailability, are probably the main responsible for the anti-inflammatory activity previously reported.

In conclusion, this work has developed a method to quantify the phenolic compounds contained in *C. citratus*; pointed the importance of harvesting and storing the plant material, in order to take the maximum advantages from the phenolic compounds use; and demonstrated, in safe doses, its anti-inflammatory activity, using an *in vivo* approach, which supports the traditional use of lemongrass infusion. Furthermore, *C. citratus* leaves flavonoids and tannins were highlighted as bioactive compounds, encouraging the development of new anti-inflammatory drugs or nutraceuticals.

Resumo

Cymbopogon citratus (DC). Stapf (Poaceae), vulgarmente conhecida como erva-príncipe, é um arbusto perene tropical originário do Sudeste Asiático. Nos países asiáticos e latino-americanos, é uma planta comummente consumida como bebida ou condimento aromático. A esta planta são lhe atribuídas propriedades antifúngicas, insecticidas, antidiabéticas, antissépticas, antimutagénicas, anticancerígenas e anti-inflamatórias. Na verdade, extratos aquosos das folhas secas são utilizados, durante todo o ano, na medicina popular para o tratamento de úlceras pépticas e problemas inflamatórios. Recentemente, alguns compostos fenólicos, tais como os glicósidos da luteolina e da apigenina e os taninos condensados, foram identificados e relacionados com efeitos antioxidantes e anti-inflamatórios. O objetivo deste trabalho foi i) validar um método analítico para a quantificação de compostos fenólicos de *C. citratus*; ii) estudar a influência da época de colheita e da qualidade da planta na composição fenólica e na atividade antioxidante do infuso usado na medicinal popular; iii) caracterizar os taninos por HPLC-PDA-ESI/MS^n; iv) validar os usos tradicionais da infusão como anti-inflamatória *in vivo*; v) obter uma formulação tópica para avaliar a permeação dos compostos fenólicos e a sua actividade anti-inflamatória; vi) traçar o perfil farmacocinético dos principais compostos fenólicos em ratos.

Três extractos - infusão (CcI), macerado com etanol a 50% (CcM50%) e macerado com etanol absoluto (CcM100%) - foram preparados e um método de HPLC-PDA, simples e eficiente, foi validado com êxito, pela primeira vez, para a identificação simultânea e quantificação dos ácidos fenólicos e flavonóides. Diferentes infusões foram também obtidas a partir de folhas colhidas em diferentes épocas (Abril, Junho, Julho, Agosto e Setembro) e diversos graus de qualidade (Alto, Médio e Baixo). Foi verificado que o conteúdo em polifenóis e a capacidade antioxidante do CcI estão fortemente relacionados com a qualidade da planta. O ensaio dos fenóis totais mostrou uma perda substancial de Agosto para Setembro. Além disso, foi possível descobrir o melhor mês para a colheita da planta no sentido de obter o máximo teor de cada grupo fenólico: Abril e Junho, para ácidos hidroxicinâmicos; Junho e Setembro, para flavonóides; e Junho, Julho e Agosto,

para taninos. Independentemente do grupo de compostos fenólicos estudado, o seu conteúdo foi sempre inversamente proporcional ao grau de envelhecimento das folhas. Para todas as espécies oxidantes testadas, as amostras de alta qualidade apresentaram os melhores resultados como antioxidantes.

A CcI foi fraccionada por cromatografia em coluna e fracções ricas em polifenóis, nomeadamente ácidos fenólicos (CcPA), flavonóides (CcF) e taninos (CcT) foram obtidos. A CcT foi caracterizada por HPLC-PDA-ESI/MS^n, revelando a presença de pro-desoxi-antocianidinas hetero-diméricas, juntamente com algumas proantocianidinas comuns. Estas estruturas flavânicas hetero-diméricas foram descritas, pela primeira vez, nesta planta e consistem em unidades de luteoliflavano ou apigeniflavano ligados a uma flavanona, naringenina ou eriodictiol, podendo apresentar-se na forma livre ou glicosilada.

CcI, CcF e CcT foram testados *in vivo*. A CcI, administrada antes e após o estímulo etanólico, reduziu significativamente a incidência e a gravidade das lesões gástricas e, consequentemente, o índice ulceroso, corroborando o uso tradicional desta planta no alívio dos sintomas da gastrite e/ou das úlceras pépticas. Por outro lado, CcI, CcF e CcT foram administrados por via oral a ratos, para avaliar o efeito anti-inflamatório no ensaio do edema da pata induzido pela carragenina. O efeito observado para a dose mais alta (68.24 mg/kg), 82.30%, foi muito semelhante ao obtido pelo fármaco de referência utilizado (diclofenac - 10 mg/kg), 84.00%. Por outro lado, as fracções CcF (7.42 mg/kg) e CcT (5.96 mg/kg) contribuíram significativamente para o efeito anti-inflamatório (59 e 61%, respectivamente).

A actividade anti-inflamatória tópica de CcI foi também estudada. Os resultados sugerem que os flavonóides, principalmente, a luteolina 7-*O*-neohesperidósido, a cassiaoccidentalin B, o carlinósido e o cinarósido, podem contribuir para o efeito anti-inflamatório tópico verificado. Formulações tópicas de CcF (0.6%), CcT (0.3%) e CcF+CcT (0.66%+0.34%) foram também testados *in vivo*, e os resultados obtidos sugerem que os taninos e flavonóides possuem também uma significativa actividade e que um sinergismo deverá ocorrer pela função das duas fracções fenólicas. Todas as amostras testadas demonstraram ausência de toxicidade *in vivo*.

A análise farmacocinética foi realizada no plasma, fígado e rim e mostraram que os compostos presentes no CcI não foram detectados *in vivo* após uma administração oral de dose única. Ao contrário, os metabolitos, luteolina 7-*O*-glucurónido e luteolina 3'-*O*-sulfato, demonstraram possuir maior biodisponibilidade, sendo provavelmente os principais flavonóides responsáveis pela actividade anti-inflamatória anteriormente descrita.

Em conclusão, este trabalho desenvolveu um método para quantificar os compostos fenólicos de *C. citratus*; apontou a importância da colheita, do armazenagem e do embalamento do material vegetal, a fim de tirar as máximas vantagens dos compostos fenólicos a utilizar; e demonstrou a sua actividade anti-inflamatória *in vivo*, sistémica e tópica, utilizando doses não tóxicas, que suporta o uso tradicional da infusão de erva-príncipe. Além disso, os flavonóides e taninos das folhas de *C. citratus* foram destacados como compostos bioativos, incentivando o desenvolvimento de novos produtos anti-inflamatórios, fitoterápicos ou nutracêuticos.

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Abbreviations

2HBA	2-Hydroxylase benzoic acid
4CL	4-Coumaroyl-CoA ligase
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
$ABTS^{\bullet+}$	ABTS cation radical
ADME	Absorption, Distribution, Metabolism, Excretion
amu	Atomic mass unit
ANOVA	Analysis of variance
ANS	Anthocyanidin synthase
AP-1	Activator protein 1
Apo E	Apolipoprotein E
Apr	April
AUC	Area under curve
$AUC_{(0\rightarrow\infty)}$	Area under curve from administration extrapolated to infinite time
$AUC_{(0\rightarrow t)}$	Observed area under curve
Aug	August
b.w.	Body weight
BAL	Bagg Albino mice
BCRP	Breast cancer resistance protein
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
C_{18}	C_{18} bonded silica stationary phase
C4H	Cinnamate-4-hydroxylase
CAF	Caffeic acid
CAM	Cell adhesion molecule
CBG	Cytosolic β-glucosidase
Cc	*Cymbopogon citratus*
CcE	*Cymbopogon citratus* infusion
CcF	Flavonoid fraction from CcI
CcI	*Cymbopogon citratus* infusion
CCI	*Cymbopogon citratus* infusion

CCL5	Chemokine (C-C motif) ligand 5
CCM100	Methanolic extract from *Cymbopogon citratus*
CCM50	50% methanolic extract from *Cymbopogon citratus*
CcT	Tannin fraction from CcI
CE	Collision energy
CES	Collision energy spread
CHI	Chalcone isomerase
CHR	Chrysoeriol
CHS	Chalcone synthase
c-Jun	Protein encoded by the JUN gene
CL	Clearance
C_{max}	Maximum concentration
CoA	Co-enzyme A
COMT	Catechol-*O*-methyltransferase
COU	*p*-Coumaric acid
COX-2	Cyclooxygenase 2 or prostaglandin-endoperoxide synthase 2
CXCL2	Chemokine (C-X-C motif) ligand 2
CXP	Cell exit potential
DAD	Diode array detector
DC.	Augustin Pyramus de Candolle (botanist)
DF_{lof}	Lack-of-fit degrees of freedom
DFR	Di-hydroflavonol reductase
DF_{ε}	Purely experimental degrees of freedom
DIO	Diosmetin
DNA	Deoxyribonucleic acid
DP	Declustering potential
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSS	Dextran sulfate sodium
EC_{50}	Half-maximal effective concentration
EP	Entrance potential
EPI	Enhanced product ion

ESI	Electro-spray ionization
ESI	Electrospray ionization
F3’5’H	Flavone 3’,5’-hydroxylase
F3’H	Flavone 3'-hydroxylase
F3H	Flavanone 3-hydroxylase
FDA	Food and Drug Administration
FF	Flavonoid fraction from CcI
FLS	Flavonol synthase
FRAP	Ferric reducing antioxidant power
FS1	Flavone synthase 1
FS2	Flavone synthase 2
FSDC	Fetal skin-derived dendritic cell line
GC	Gas chromatography
GT	Glycosyltransferase
H	High
HE	Hematoxylin and eosin
HFA	Human flora-associated
HPLC	High-performance liquid chromatography
I	Number of i concentration levels
i.p.	Intraperitoneal
ICAM	Intercellular adhesion molecule
ICH	International Conference on Harmonization
ICR	Imprinting Control Region mice
IgE	Immunoglobulin E
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ISO	Isoorientin
ISV	Isovitexin
IV	Intravenous
IκB	NF-κB activators family

J	Total number of *j* injections per *i* concentration levels
JNK	c-Jun NH_2-terminal kinase
Jul	July
Jun	June
k	Elimination rate constant
L	Low
L3'S	Luteolin 3'-*O*-sulfate
L7G	Luteolin 7-*O*-glucuronide
LAR	Leucoanthocyanidin reductase
LC	Liquid chromatography
LD50	Lethal dose, 50%
LDL	Low-density lipoprotein
LDOX	Leucoanthocyanidin oxidase
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LPH	Lactase phlorizin hydrolase
LPS	Lipopolysaccharide
LT	Leukotriene
LTB_4	Leukotriene B_4
LUT	Luteolin
M	Medium
m/z	Mass-to-charge ratio
MAPK	Mitogen-activated protein kinase
MIP-2	Macrophage inflammatory protein 2
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
MRT	Mean residence time
MS	Mass spectrometry

MS^2	Tandem mass spectrometry
MW	Molecular weight
n.a.	Not applicable
n.d.	Not detected
NC	Not calculated
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
$NO^{\bullet}$	Nitric oxide radical
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSAID	Non-steroid anti-inflammatory drug
*O*MTS	*O*-Methyltransferases
p.o.	*Per os*
p38	Protein from the MAPK family
p65	Protein from the NK-KB family
PAL	Phenylalanine ammonia lyase
PBS	Phosphate buffer saline
PCA	Protocatechuic acid
PDA	Photo-diode array
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PPAR-α	Peroxisome proliferator-activated receptor α
PPAR-γ	Peroxisome proliferator-activated receptor γ
QM	Quinone methide cleavage
RANTES	see CCL5
RAW 264.7	Abelson murine leukemia virus-transformed macrophages
RDA	Retro Diels-Alder reaction
RER	Rough endoplasmic reticulum

RP	Reverse phase
RSD	Relative standard deviation
RUT	Rutin
SD	Standard deviation
SEM	Standard error of the mean
Sep	September
SER	Smooth endoplasmic reticulum
SPE	Solid-phase extraction
SS_{lof}	Lack-of-fit sum squares
SS_{ε}	Purely experimental sum squares
STAT	Signal transducers and activators of transcription
SULT	Sulfotransferase
$T_{1/2}$	Half-life time
$t_{1/2}$	Half-life time
Tb	Mean reaction time of blank rats
TEAC	Trolox®-equivalent antioxidant capacity
TEM	Transmission electron microscopy
TF	Tannin fraction from CcI
TLC	Thin layer chromatography
t_{max}	Maximum time
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF-α	Tumor necrosis factor α
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TPA	Texture profile analysis
TPTZ	2,4,6-Tripyridyl-S-triazine
Tt	Mean reaction time of treated rats
Tyk2	Tyrosine-protein kinase 2
U.I.	Ulcer index
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase
UPLC	Ultra-performance liquid chromatography

UV	Ultraviolet radiation
UV-A	A-type ultraviolet radiation
UV-B	B-type ultraviolet radiation
V_c	Mean variation of the paw volume in control rats
VCAM	Vascular cell adhesion molecule
V_d	Volume of distribution
Vt	Mean variation of the paw volume in treated rats
Wc	Average number of writhes in control rats
Wt	Average number of writhes in test rats
$\hat{y}_i$	Estimated experimental response for the level *i*
$\bar{y}_i$	Average response for the level *i*
σ_{lof}^2	Lack-of-fit variance
σ_{ε}^2	Purely experimental variance

INTRODUCTION

CHAPTER I

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*equally contributing authors

A. *Cymbopogon citratus*

1. Botanic characterization

1.1 Classification and etymology

The accepted name *Cymbopogon citratus* (DC.) Stapf (**Table I.1**) was originally described in 1906 and published in the *Bulletin of Miscellaneous Information* (see **Attach**). The genus *Cymbopogon* belongs to the family Poaceae (formerly Gramineae). Poaceae encompasses approximately 800 genera and 10.000 species (Watson and Dallwitz, 1992). The economic importance of this family is unquestionable for it has many different types of use, and it includes cereals, sugarcane, fodder and pasture, bamboo, etc.

The genus *Cymbopogon* includes about 70 species distributed throughout the tropics and subtropics of Africa, Asia and Australia, predominantly in Asia, having been introduced in tropical America (Shouliang and Phillips, 2006). The name of the genus *Cymbopogon* derives from the Greek "kymbe" meaning boat and "pogon" (beard), for the arrangement of the inflorescence, and the epithet "citratus" derives from the ancient Latin "citrus" related to the lemon-scented of the leaves.

1.2 Synonyms and common names

There is a quite large list of scientific names used in the past to refer this species, the synonyms. Following the most recent and reliable revision of the species, the synonyms are listed below:

Andropogon citratus DC., Cat. Pl. Horti Monsp. 78. 1813 [basionym].
Andropogon cerifer Hack., Fl. Bras. 2(4): 281 1883.
Andropogon citriodorus Desf., Tabl. École Bot. ed. 2: 15 1815.
Andropogon fragrans C.Cordem., Fl. Réunion 122 1895.
Andropogon nardus subsp. *ceriferus* (Hack.) Hack. Monogr. Phan. 6: 605 1889.
Andropogon roxburghii Nees ex Steud. Syn. Pl. Glumac. 1: 395 1854.

Table I.1. Taxonomic hierarchy of *Cymbopogon citratus* (DC.) Stapf (Integrated Taxonomic Information System, 2015).

Kingdom	**Plantae (plants)**
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta (land plants)
Superdivision	Embryophyta
Division	Tracheophyta (vascular plants; tracheophytes)
Subdivision	Spermatophytina (seed plants; spermatophytes)
Class	Magnoliopsida
Superorder	Lilianae (monocots; monocotyledons)
Order	Poales
Family	Poaceae (grasses)
Genus	*Cymbopogon* Spreng.
Species	*Cymbopogon citratus* (DC.) Stapf

This information is very important because often scientific papers concerning chemical characterization, ethnopharmacology and biological activities could be referred to *Cymbopogon citratus* (DC.) Stapf but under the name of a synonym.

The common name given to a species is another important question to ascertain the identity of each plant. In the various vernacular languages, the species *C. citratus* is known by a multitude of different names, sometimes leading to uncertain identification. In **Table I.2** are some examples of common names attributed to *C. citratus* in several countries.

Table I.2. Common names used in different countries for the *Cymbopogon citratus*.

Country(ies)	Common name(s)
Angola	"Chá de caxinde"
Brazil	"Capim-limão", "Capim-santo", Capim-cidreira"
China	香茅
English-speaking countries	"Lemongrass", "Citronella", "Squinant"
Ethiopia	"Tej-sar"
France	"Citronelle", "Verveine des Indes"
Germany	"Zitronengrass"
India	"Sera", "Verveine"
Indonesia	"Sereh", "Tanglad"
Israel	"Limonit"
Italy	"Citronella", "Cimbopogone"
Japan	レモングラス
Korea	레몬 그라스
Malaysia	"Sakumau"
Mexico	"Zacate-limón"
Netherlands	"Citroengras"
Portugal	"Erva-príncipe"
São Tomé e Príncipe	"Fiá-xalela"
Spain	"Hierba-luisa", "Hierba-limón", "Limoncillo"
Sweden	"Citrongräss"
Thailan	"Ta-khrai"
Turkey	"Limon otu"
Venezuela	"Citronera"

1.3 Botanic description

C. citratus is native in Sri Lanka and South India, being widely cultivated in tropical Asia and South America.

It is a tropical grass, shortly rhizomatous, that grows in dense clumps, up to 1.8 m in height and about 1.2 m in width (**Figure I.1**). The leaves are evergreen, bright bluish-green and release a citrus aroma when crushed. They are strap-like, 1.3-2.5 cm wide, about 0.9 m long, ending by drooping tips. The flowers are arranged in inflorescences (panicles), 30-60 cm long, however, the plants that are more likely to find are cultivars and do not typically produce flowers. The fruit is an oblong caryopsis (**Figure I.2**) (Metcalfe, 1960).

Figure I.1. *Cymbopogon citratus* (http://zipcodezoo.com/index.php/Cymbopogon_citratus).

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 is formed by 1-2 cell layers under the upper epidermis. The vascular bundles, of about 3-4 layers of sclerenchyma, are embedded in chlorenchyma cells forming a Kranz structure (Metcalfe, 1960).

Figure I.2. (A) Botanical illustration of *Andropogon citratus* DC., basionym of *Cymbopogon citratus* (DC.) Stapf, representing the structures of the plant: 1. young plant; 2. lower portion of adult plant; 3. adult leaves; 4. inflorescence (panicle); 5. spikelet; 6. flower (floret); 7. lower glume; 8. upper glume; 9. lemma; 10. palea (*Bulletin of Miscellaneous Information*, 1906). **(B)** Herbarium specimen of *Cymbopogon citratus* (DC.) Stapf collected in Portugal and deposited at the Herbarium of Aromatic and Medicinal Plants of the Faculty of Pharmacy – University of Coimbra (A. Figueirinha 0109).

2. Ethnopharmacology

The use of the plant in folk medicine occurs in almost all continents although the largest source of ethnopharmacological information on the *C. citratus* is originally from tropical countries of Africa, Asia and South American, covering a wide range of applications. Of these, stands out its use in the treatment of respiratory, gastric and nervous system problems.

C. citratus has been used in various formulations alone or in association with other plants and administered externally or orally. It is predominantly used in the form of infusion or decoction of leaves fresh and dried (Arrey Tarkang *et al.*, 2013; Carbajal *et al.*, 1989; Hilgert, 2001; Leite *et al.*, 1986; Lorenzetti *et al.*, 1991; Melo *et al.*, 2001; Shah *et al.*, 2011; Soares *et al.*, 2013), or through its the essential oil (Gupta, 1995; Ross, 2010). Sometimes the plant is also used in bathing (Coelho-Ferreira, 2009; Ruysschaert *et al.*, 2009; Zumsteg and Weckerle, 2007). The root is less used than the leaves, often in the form of decoction. In Brazil, folk medicine discourages the intake of the root extracts by pregnant, since causes uterine contractions (Rodrigues, 2007). There are numerous sources of information gathering ethnomedicinal studies performed in the course of the twentieth century. The most relevant ethnopharmacological studies are presented in **Table I.3.**

Table I.3. Ethnopharmacological studies on *Cymbopogon citratus* (cont.).

Country	Extract	Uses	Reference
Argentina	Leaf infusion	Colds and cough	Hilgert, 2001
	Leaf decoction	Stomachic, hypotensive and cardiotonic	Scarpa, 2004
	Leaf placed directly	Toothaches	
Bolivia	Leaf decoction	Stomach pain, swellings by freezing cold and tranquilizer	Macía *et al.*, 2005
Brazil	Leaf infusion	Diarrhea, bad cold, headache, muscular pain, rheumatism, fever, hypertension, blood circulation, general pains and sedative	de Albuquerque *et al.*, 2007
	Leaf decoction	Bad cold and rheumatism	Di Stasi *et al.*, 2002
	Root infusion	Antidiuretic	
	Bath	Flu with scratching throat	Coelho-Ferreira, 2009
China	Bath	Relieve pain	Au *et al.*, 2008
Costa Rica	Leaf	Cough, carminative, expectorant and depurative	Morton, 1981
Cuba	Leaf infusion	Hypertension, catarrh and rheumatism	Carbajal *et al.*, 1989
	Decoction	Nervousness, insomnia and fever	Cano and Volpato, 2004
Democratic Republic of Congo	Leaf decoction	Cough, stomach problems, diarrhea, fever, malaria, oedema and to stimulate digestion	Mesia *et al.*, 2007
Egypt	Leaf infusion	Renal antispasmodic and diuretic	Locksley *et al.*, 1982
Equator	Leaf infusion	Gastritis, relaxant, stomach, pain and diarrhea	Tene *et al.*, 2007
Ethiopia	Root	Stomachache	Teklehaymanot *et al.*, 2007
Ghana	Poultice	Boils, swellings	Agyare *et al.*, 2009
French Guiana	Leaf (with other plants)	Antimalarial	Vigneron *et al.*, 2005
Honduras	Leaf decoction	Postpartum abdominal pain and lactation	Ticktin and Dalle, 2005
India	Oil	Carminative	Poonam and Singh, 2009
	Leaf	Stimulant, sudorific, antiperiodic and anti-catarrhal	Khare, 2007
	Essential Oil	Carminative, analgesic, antipyretic, antibacterial and antifungal	
Indonesia	Essential Oil	Sedative, antiseptic	Zumsteg and Weckerle, 2007
Malaysia	Leaf infusion	Emmenagogue	Burkill *et al.*, 1936
Mexico	Leaf infusion	Influenza and gastrointestinal disorders	Andrade-Cetto, 2009
	Leaf decoction	Cough	Giovannini and Heinrich, 2009

Table I.3. Ethnopharmacological studies on *Cymbopogon citratus*.

Country	Extract	Uses	Reference
Nepal	Pounded plant and juice taken with hot water	Cold	Shrestha and Dhillion, 2003
Nicaragua	Leaf infusion	Abdominal and back pain, postpartum abdominal pain, promote lactation, fever, digestive ailments (flatulence, heartburn and stomachache)	Coe, 2008
	Root liniment	Backache	
Nigeria	Leaf decoction	Antimalarial	Ajibesin *et al.*, 2007
		Yellow fever	Ajaiyeoba *et al.*, 2003
Peru	Leaf	Antimalarial, anti-leishmania	Kvist *et al.*, 2006
Philippines	Leaf infusion	Indigestion and stomach problems, anti-stress, colds, fevers, pain and arthritis	Dagupen *et al.*, 2011
Portugal	Leaf infusion	Gastric analgesic, digestive system, gall-bladder ailments, intestinal anti-inflammatory, sea-sickness, renal antispasmodic and bladder ailments	Novais *et al.*, 2004
Suriname	Bath	Baby´s abdominal pain, fever, cold and viral infections	Ruysschaert *et al.*, 2009
Thailand	Leaf or stem	Carminative, diuretic, antipyretic	Wannissorn *et al.*, 2005
Trinidad & Tobago	Leaf or root	Cold, flu, fever and diabetes	Mahabir and Gulliford, 1997
	Leaf and root	Cough, cold, fever	Clement and Seaforth, 2015
Uganda	Leaf aqueous extract (with other plants)	Antimalarial	Tabuti, 2008
	Leaf	Influenza	Tabuti *et al.*, 2003
United States of America	Leaf infusion	Externally for healing wounds and bone fractures	Spring, 1989

3. Phytochemistry

The non-volatile constituents of *C. citratus* have been much less studied because of the higher economic interest so far has been attributed to the essential oil. The literature review highlights the existence of a large variability in chemical composition of the fixed constituents of *C. citratus*. Isolation of luteolin, luteolin 7-*O*-glucoside (cynaroside), isoscoparin and 2"-*O*-rhamnosyl isoorientin from the leaves and rhizomes of *C. citratus* has been reported (Avoseh *et al.*, 2015). Other phenolic compounds referred to aerial parts of *C. citratus* are quercetin, kaempferol

and apigenin, catechol, chlorogenic acid, caffeic acid and hydroquinone (Faruq, 1994; Miean and Mohamed, 2001; Tapia *et al.*, 2007). It is observed, in particular, predominance of flavone glycosides (luteolin and apigenin) and seldom their aglycones (Cagiotti *et al.*, 2001). Only one author refers to the presence of kaempferol as the only flavonoid present, although not specify whether it is the aglycone or a glycoside (Miean and Mohamed, 2001), while in another study the presence of methylated aglycones was cited (Cheel *et al.*, 2005).

The prevalence of flavonoids, mainly flavones and their *C*- and *O*-type glycosides, and phenolic acids, is a characteristic feature common to other species of the same family (Poaceae) (Harborne and Williams, 1976). Some authors identified both types of glycosides (*C*- and/or *O*-) (Figueirinha *et al.*, 2008; Gunasingh and Nagarajan, 1981 Orrego *et al.*, 2009; Tapia *et al.*, 2007). The positions of glycosylation at *C*-glycosides were identified in C6 and C8, while in the case of the *O*-glycosides, the sugar portion was bound in position O7 (Cheel *et al.*, 2005; De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008). These differences may be originated by different environmental conditions in which the plant grew. Glycosides have been identified with more than two sugars, these being often glucose and rhamnose (Cheel *et al.*, 2005; De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008; Gunasingh and Nagarajan, 1981).

Phenolic acids are most often identified phenolic compounds in the plant leaves, especially being the hydroxycinnamic type (caffeic, chlorogenic and *p*-coumaric) (Cheel *et al.*, 2005; De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008; Tapia *et al.*, 2007), but also hydroxybenzoic acids (Tapia *et al.*, 2007). Marques *et al.* (2008) identified and quantified various isomers of derivatives of hydroxycinnamic acids in infusions and methanolic extracts: isomers 3, 4 and 5 of caffeoylquinic acid, representing the 5-isomer, 53% of total phenolic acids identified. Also identified the isomers 3, 4 and 5-feruloylquinic acid, isomers 3 and 4; and 3, 5 and 4, 5-dicaffeoylquinic acid. This study did not detect caffeic acid nor gallic acid (Marques and Farah, 2009). Another study on the phytochemical composition of *C. citratus* also revealed the presence of tannins,

however, very little effort has been made in the isolation of these compounds despite the appreciable amounts reported through quantitative phytochemical tests. Figueirinha and collaborators (2008) fractionated extracts of the species cultivated in Portugal and reported about 4.35% (dry weight) of condensed tannins, while *C. citratus* from Nigeria showed about 0.6% of tannins (Asaolu *et al.*, 2009). *C. citratus* is the single species of *Cymbopogon* which is most exploited for its tannin content (Avoseh *et al.*, 2015). Nevertheless, at present there is no data that characterize the chemical structure of this class of phenolic compounds.

Other compounds have been studied in *C. citratus* leaves; two terpenoids (cymbopogonol and cymbopogone) were identified in its coating (Avoseh *et al.*, 2015) and the wax presence was studied with the aim of establishing grasses classification criteria (Maffei, 1996), while in plant rhizomes alkaloids and saponins were detected (Negrelle and Gomes, 2007). Another recent studies revealed the presence of other minor groups of phytochemicals, namely alkaloids, saponins, anthraquinones and steroids in lemongrass leaves (Asaolu *et al.*, 2009; Oloyede, 2009; Soares *et al.*, 2013). More recently, it has been reported the existence of phenolic compounds with medicinal properties in the hydrodistillation water of lemongrass leaves (Tavares *et al.*, 2015). **Table I.4** summarizes the non-volatile compounds identified in extracts of *C. citratus*.

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus* (cont.).

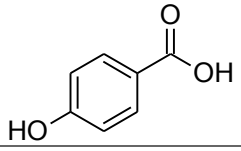
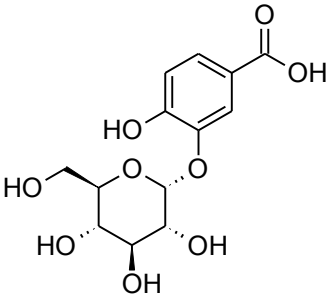
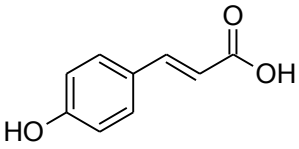
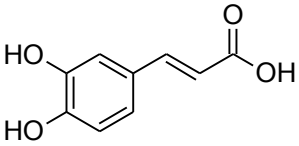
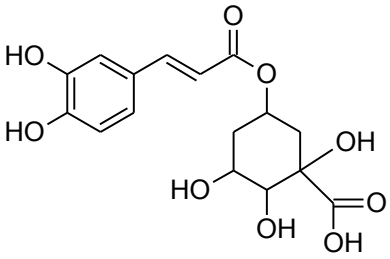
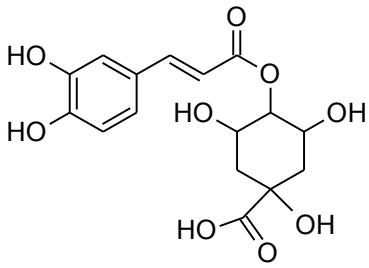
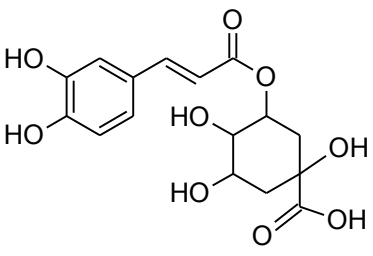
Compounds Identified	Structure	Reference
Phenolic acids		
p-Hydroxybenzoic acid		Tapia *et al.*, 2007
p-Hydroxybenzoic acid 3-*O*-glucoside		Tapia *et al.*, 2007
p-Coumaric acid		De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008
Caffeic acid		Cheel *et al.*, 2005; De Matouschek and Stahl-Biskup, 1991; Tapia *et al.*, 2007
3-*O*-Caffeoylquinic acid		Cheel *et al.*, 2005; Marques and Farah, 2009; Tapia *et al.*, 2007
4-*O*-Caffeoylquinic acid		Marques and Farah, 2009
5-*O*-Caffeoylquinic acid		Marques and Farah, 2009; Tapia *et al.*, 2007

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus* (cont.).

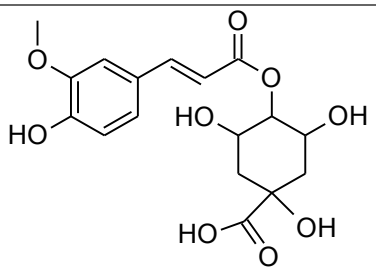
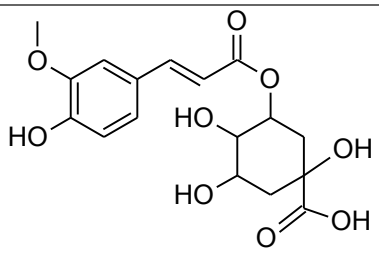
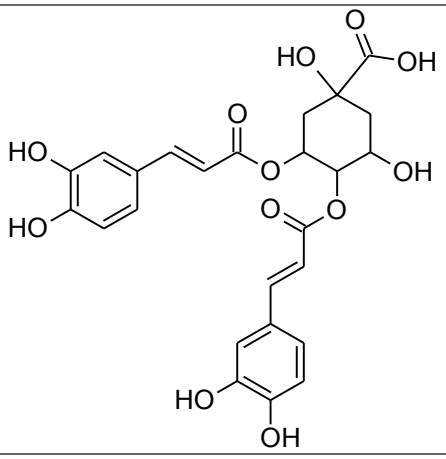
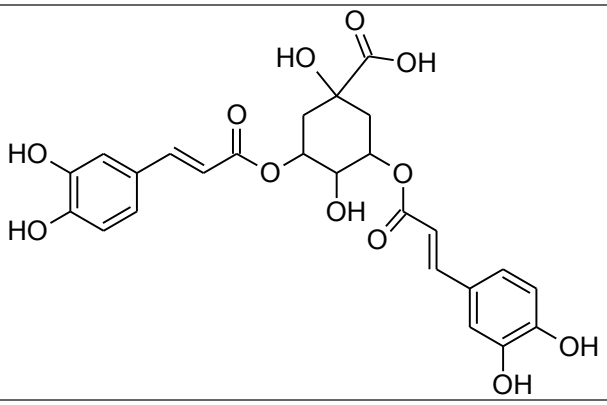
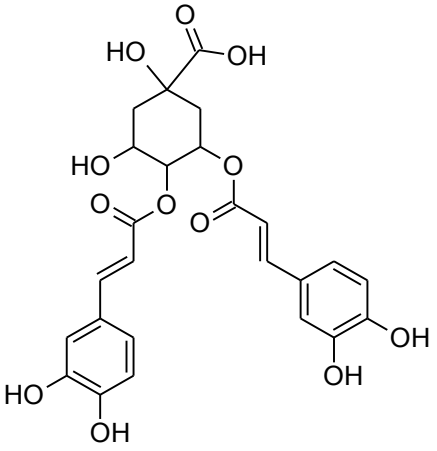
Compounds Identified	Structure	Reference
4-*O*-Feruloylquinic acid		Marques and Farah, 2009
5-*O*-Feruloylquinic acid		Marques and Farah, 2009
3,4-*O*-Dicaffeoylquinic acid		(Marques and Farah, 2009)
3,5-*O*-Dicaffeoylquinic acid		Marques and Farah, 2009
4,5-*O*-Dicaffeoylquinic acid		Marques and Farah, 2009

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus* (cont.).

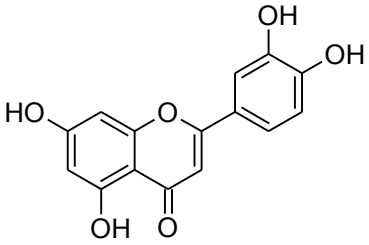
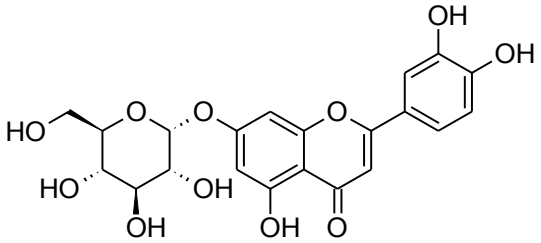
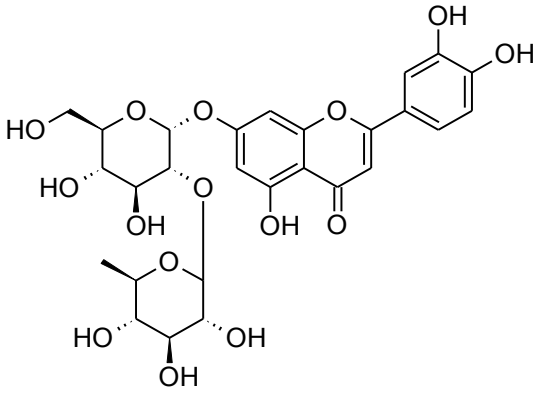
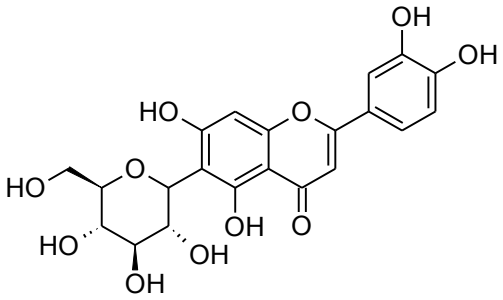
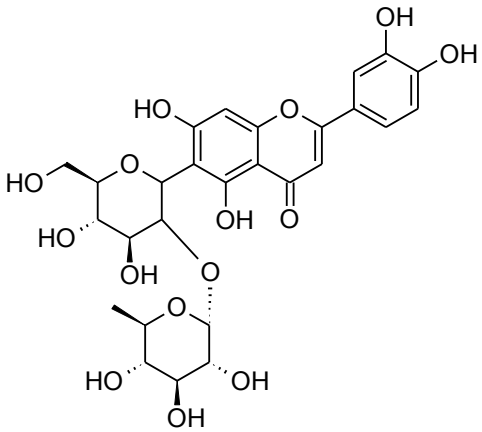
Compounds Identified	Structure	Reference
Flavonoids		
Luteolin		Cagiotti *et al.*, 2001; De Matouschek and Stahl-Biskup, 1991
Luteolin 7-*O*-glucoside (Cynaroside)		Figueirinha *et al.*, 2008; Gunasingh and Nagarajan, 1981
Luteolin 7-*O*-neohesperidoside		De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008
Luteolin 6-*C*-glucoside (Isoorientin)		Cheel *et al.*, 2005; De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008; Gunasingh and Nagarajan, 1981
Luteolin (2''-*O*-rhamnosyl)-6-*C*-glucoside		Cheel *et al.*, 2005; De Matouschek and Stahl-Biskup, 1991; Figueirinha *et al.*, 2008

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus* (cont.).

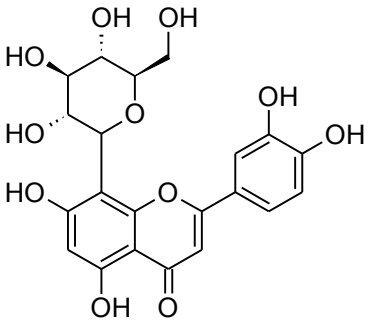
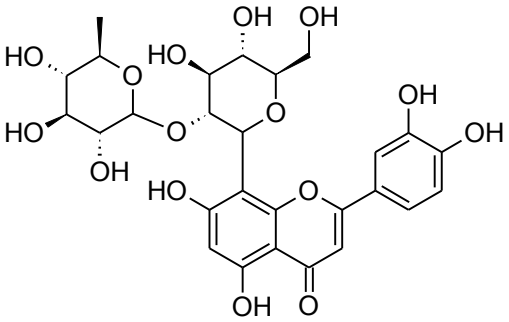
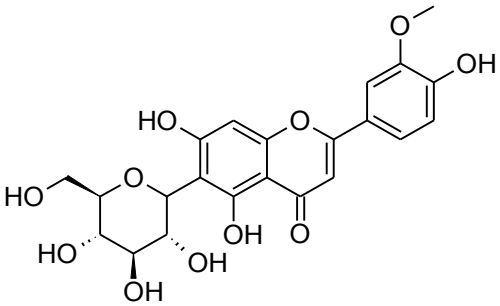
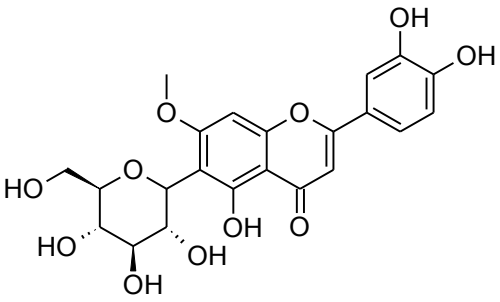
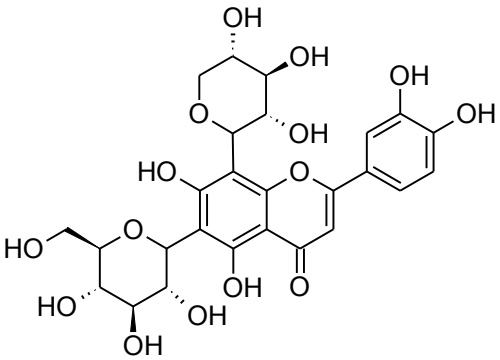
Compounds Identified	Structure	Reference
Luteolin 8-*C*-glucoside (Orientin)		Cheel *et al.*, 2005
Luteolin (2''-*O*-rhamnosyl)-8-*C*-glucoside		De Matouschek and Stahl-Biskup, 1991
Chrysoeriol 6-*C*-glucoside (Isoscoparin)		Cheel *et al.*, 2005
7-Mehtoxyluteolin 6-*C*-glucoside (Swertiajaponin)		Cheel *et al.*, 2005
Luteolin 6-*C*-glucosyl-8-*C*-arabinoside (Carlinoside)		Figueirinha *et al.*, 2008

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus* (cont.).

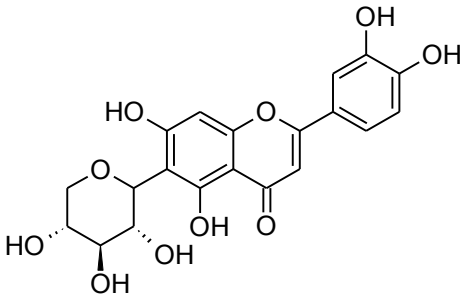
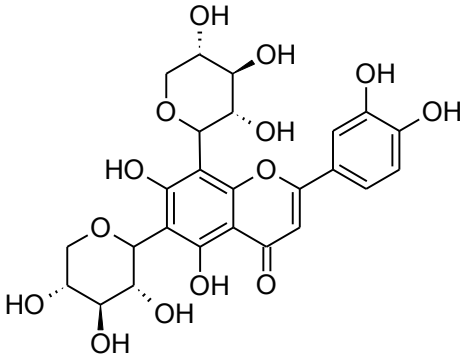
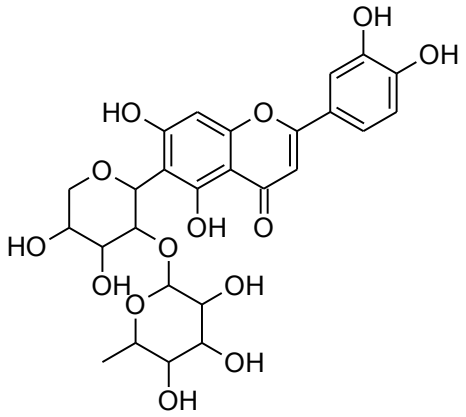
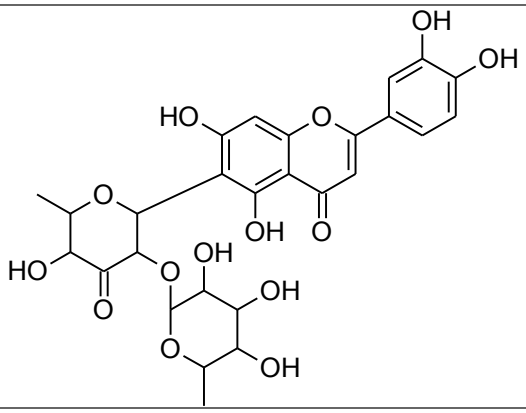
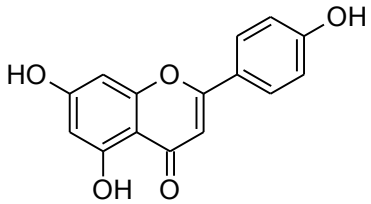
Compounds Identified	Structure	Reference
Luteolin 6-*C*-pentoside		Figueirinha *et al.*, 2008
Luteolin 6-*C*-pentosyl-8-*C*-pentoside		Figueirinha *et al.*, 2008
Luteolin 6-*C*-pentosyl-8-*C*-deoxyhexoside (Kurilensin A)		Figueirinha *et al.*, 2008
Luteolin 6-*C*-(6-deoxyribo-hexos-3-ulosyl)-2"-*O*-rhamnoside (Cassiaoccidentalin B)		Figueirinha *et al.*, 2008
Apigenin		Miean and Mohamed, 2001

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus* (cont.).

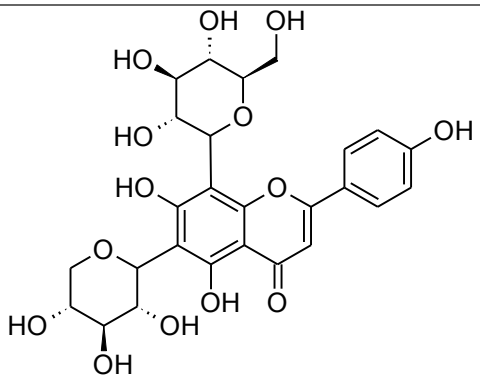
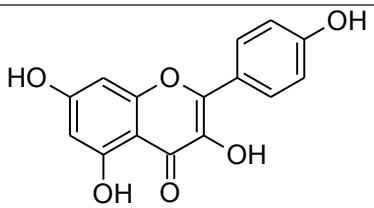
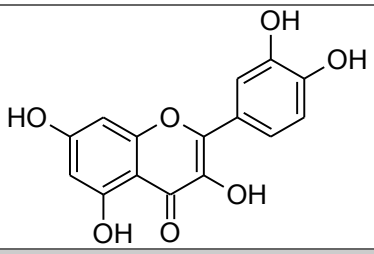
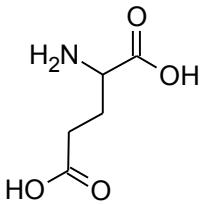
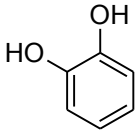
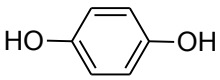
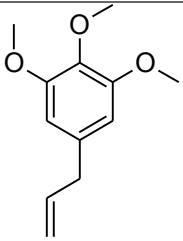
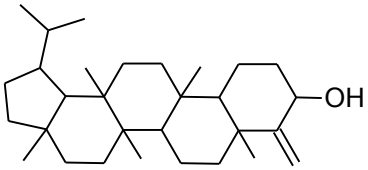
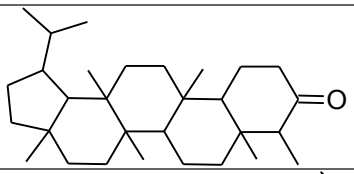
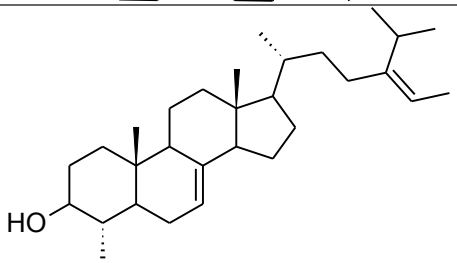
Compounds Identified	Structure	Reference
Apigenin 6-*C*-arabinosyl-8-*C*-glucoside (Isoschaftoside)		Figueirinha *et al.*, 2008
Kaempferol		Miean and Mohamed, 2001
Quercetin		Miean and Mohamed, 2001
Others		
Glutamic acid		Tapia *et al.*, 2007
Catechol		Faruq, 1994
Hydroquinone		Faruq, 1994
Elemicin		Faruq, 1994
Cymbopogonol		Avoseh *et al.*, 2015

Table I.4. Non-volatile compounds identified in *Cymbopogon citratus*.

Compounds Identified	Structure	Reference
Cymbopogone		Avoseh *et al.*, 2015
α-Sitosterol		Negrelle and Gomes, 2007

4. Anti-inflammatory properties

The anti-inflammatory activity is often referred to *C. citratus* in ethnopharmacological studies. Despite this fact, has not been carried out much work with extracts, both *in vitro* and *in vivo*. In addition, results are often contradictory, eventually due to the complexity of the mechanisms involved in the inflammatory response, and also to the methodological approaches to assess this activity.

In vitro studies usually evaluate the activity on the production of various mediators of inflammation in cell lines. Typically, it is determined the ability of the extracts to capture the radical nitric oxide (NO•) released by the cells stimulated with an inflammatory agent. An infusion of *C. citratus* inhibited NO• radical produced by RAW 264.7 macrophages activated with lipopolyssacharide (LPS), even though to a lesser extent than green tea (Tsai *et al.*, 2007).

The action on the production of pro-inflammatory and anti-inflammatory cytokines by cells of the immune system is often also studied. A 70% methanolic extract of *C. citratus* applied in BALB/c mouse macrophages inhibited the production of pro-inflammatory IL-1β and induced the production of anti-inflammatory IL-6 cytokines. The essential oil also had the same effect on the production of these cytokines (Sforcin *et al.*, 2009). Another recent work demonstrated the capacity of a lemongrass 50% ethanol extract to suppress some

pro-inflammatory mediators in LPS-stimulated murine alveolar macrophages (Tiwari *et al.*, 2010). A lemongrass decoction has been reported as having a beneficial effect in ameliorating murine spontaneous ileitis, by decreasing lymphocyte recruitment to the inflamed intestine (Watanabe *et al.*, 2010). Recently, a work has been published, reporting the inhibition of IL-1β production by a methanolic extract from *C. citratus* in LPS-induced human peripheral blood mononuclear cells (Salim *et al.*, 2014).

The infusion of *C. citratus* continues to be the most studied polar extract. It has been described to have an anti-inflammatory effect on LPS-stimulated dendritic cells (FSDC), and this activity is mainly attributed to its polyphenols, mono- and polymeric flavonoids, luteolin glycosides and condensed tannins, respectively. The same study proved that lemongrass infusion was able to scavenge NO and inhibit iNOS expression *in vitro* (Figueirinha *et al.*, 2010). Moreover, the same extract was used in RAW 264.7 macrophages, and demonstrated inhibition on iNOS expression, NO production and various LPS-induced pathways, including p38 mitogen-activated protein kinase (MAPK), c-jun NH_2-terminal kinase (JNK)1/2 and the transcription nuclear factor (NF)-κB activation; and also the PGE_2 production (Francisco *et al.*, 2011). Another aqueous extract (hydrodistillation water, a waste obtained from essential oil extraction) was also investigated and its phenolic composition was found to be similar to the infusion. Curiously, this hydrodistillation aqueous extract was able to inhibit the NO production in LPS-induced RAW 264.7 macrophages as well. Therefore, we may speculate that this kind of anti-inflammatory activity is tightly linked to the nature of the phenolic compounds present in the plant (Tavares *et al.*, 2015).

In *in vivo* studies, the anti-inflammatory activity is typically tested in mice, in which the inflammation is induced. The results are then evaluated by observation of macroscopic signals, but certain inflammatory markers (e.g. cytokines from immune cells) can also be quantified. The very first study reporting the anti-inflammatory activity of a lemongrass polar extract was achieved using a decoction, orally administrated to rats, 5h after the inflammatory stimulus by carrageenan. The extract exhibited an oedema reduction of 20% (Carbajal *et al.*,

1989). In another *in vivo* study for anti-inflammatory activity evaluation in mice, using the induced granuloma model, it was demonstrated that a 30% ethanol extract, administered orally, showed no anti-inflammatory activity (Rodríguez *et al.*, 1996). However, the oral administration of a decoction during two weeks to mice with chronic intestinal inflammation, lead to an improvement of colitis and reverted the weight loss. In addition to the improvements in histological examination, there was a smaller amount of T lymphocytes in the mucosa and an inhibition of the expression of integrin β7, which is responsible for the migration of leukocytes to the site of inflammation (Watanabe *et al.*, 2008). Another study showed that oral administration of a hexane extract reduced the leukocytes/eosinophils in Bagg Albino mice (BAL), the eosinophil peroxidase activity in BAL, the infiltration of leukocytes in lung tissue, the production of mucus in the respiratory tract, the level of IL-4 in BAL and the nuclear expression of NF-κB (Santos *et al.*, 2015). A recent work suggests that lemongrass possess biologically active compounds with antipyretic, anti-inflammatory and antinociceptive activities, useful in the symptomatic treatment of malaria fever (Tarkang *et al.*, 2015).

C. citratus extracts are also used in the treatment of gastro-intestinal disorders. The diversity of symptoms for which it is indicated in traditional medicine suggests that the use of this plant is rather complex, either in the aetiology or in the likely mechanisms of action. However, *in vivo* or *in vitro* studies in the literature are very scarce. In the light of the studies in this field, the action of *C. citratus* on the digestive tract may be in part related to the antimicrobial activity against *Helicobacter pylori*, one of the triggering agents of gastric ulcers (Bergonzelli *et al.*, 2003; Ohno *et al.*, 2003) or its local anti-inflammatory action (Watanabe *et al.*, 2008). Regarding the adverse effects, it was found that infusion administration, in mice, from twenty to one hundred times more concentrated than preparations for human consumption, did not cause any gastric alterations when administered orally, but did so intraperitoneally, in which it was observed a reduction of the intestinal transit (Leite *et al.*, 1986).

In summary, the anti-inflammatory activity studies using extracts from *C. citratus* are scarce and have contradictory results. This may be due to several factors such as the composition of the extracts, plant origin or even the diverse inflammation models.

5. Toxicity

With regard to *in vivo* studies, the literature is scarce and mostly oriented towards the essential oil. The toxicity of *C. citratus* hydroalcoholic extracts was evaluated *in vivo*, using albino rats. It was obtained a LD50 value of 460 mg/kg (Parra *et al.*, 2001). On the other hand, using an infusion obtained from dried leaves, a dose about 20 times higher than normally used, administered orally for a period of 2 months to one group of rats which also included healthy pregnant females did not show any toxic effects (Formigoni *et al.*, 1986).

Clinical studies in humans involving the use of extracts are even scarcer. An infusion was administered orally for 2 weeks, in healthy volunteers, and after an evaluation of different blood and urine parameters was carried out. Only a slight increase was observed in direct bilirubin and amylase in some volunteers, without any clinic manifestation. However, these results do not assign any effect by administration directly of the infusion (Leite *et al.*, 1986).

There are also reports on the occurrence of dermatitis caused by contact with dry leaves of *C. citratus*, and this effect is usually attributed the constituents of the essential oil (Ross, 2010). In fact, citral, even diluted at 50%, can cause erythema when placed on the skin. In general, it should be avoided the contact of aldehydes with the mucous membranes (Buckle, 2001).

B. Phenolic Compounds

1. Biosynthesis

All plant constituents derive from the metabolism of carbohydrates synthesized during photosynthesis from CO_2 and H_2O. In the case of phenolic compounds, their synthesis takes place essentially in two pathways: shikimic acid and malonate (Vermerris and Nicholson, 2006) (**Figure I.3**).

The shikimate pathway, common in plants and microorganisms, consists in the synthesis of 3-dehydroshikimic acid from the glycolysis of phosphoenolpyruvate and from erythrose 4-phosphate which comes from the pentose phosphate pathway. The 3-dehydroshikimic acid is then transformed into shikimic acid, which may also be converted into *β*-glucagon, which in turn is converted into *penta-O*-galloylglucose, a key intermediate in the synthesis of gallotannins and ellagitannins (Niemetz and Gross, 2005). On the other hand, shikimic acid can lead to the synthesis of chorismate thus participates in the pathway of aromatic amino acids, like tryptophan, phenylalanine and tyrosine (Vermerris and Nicholson, 2006). Phenylalanine formed by the shikimate pathway originates another important biosynthetic pathway of phenolic compounds, specifically phenolic acids and flavonoids and derivatives: the phenylpropanoids pathway (Crozier *et al.*, 2006; Grotewold, 2008; Vermerris and Nicholson, 2006).

The phenylpropanoids pathway (**Figure I.4**) begins with phenylalanine deamination catalysed by the enzyme phenylalanine ammonia lyase (PAL), producing the cinnamic acid.

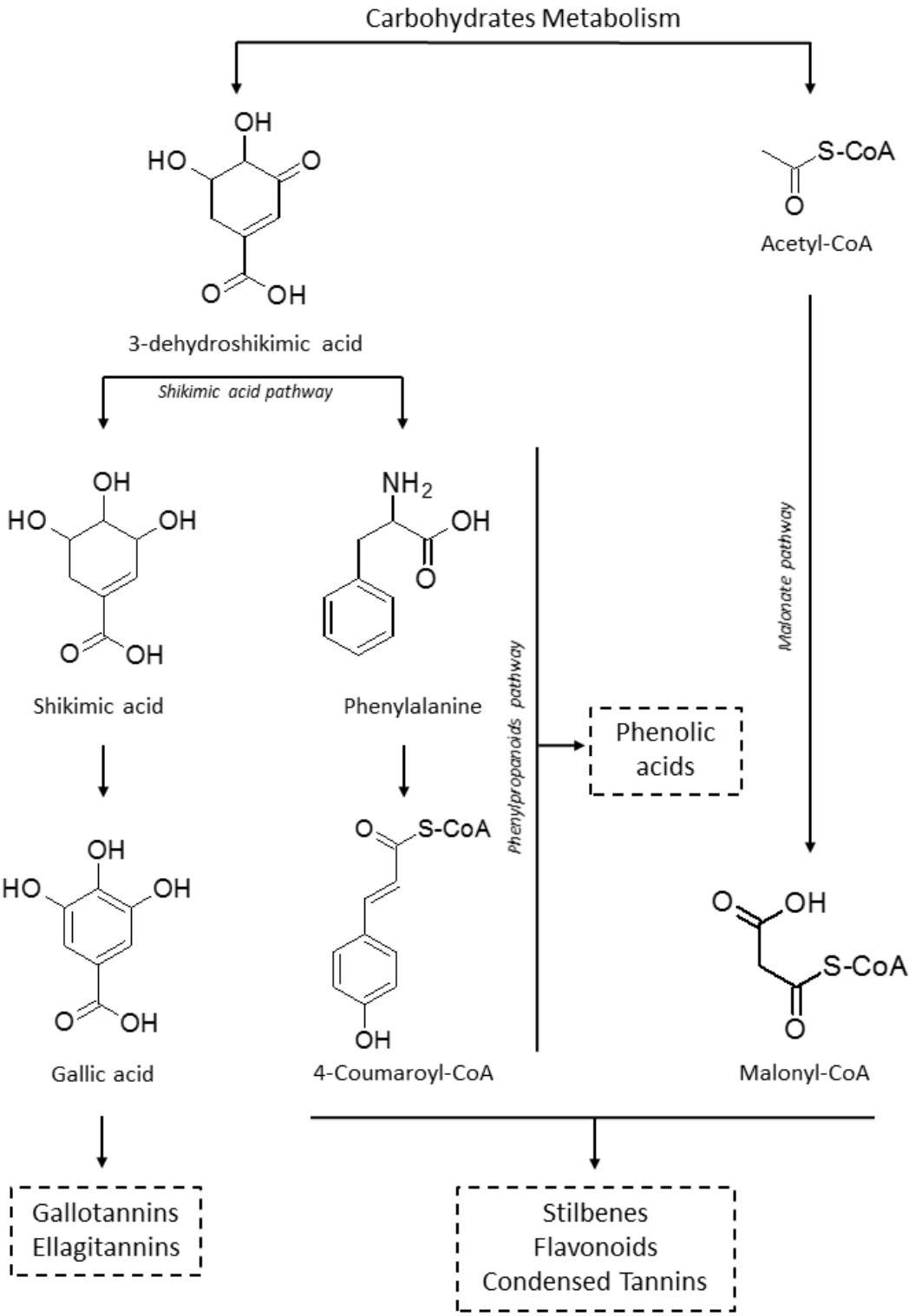


Figure I.3. Shikimate and malonate pathways (adapted from Vermerris and Nicholson, 2006).

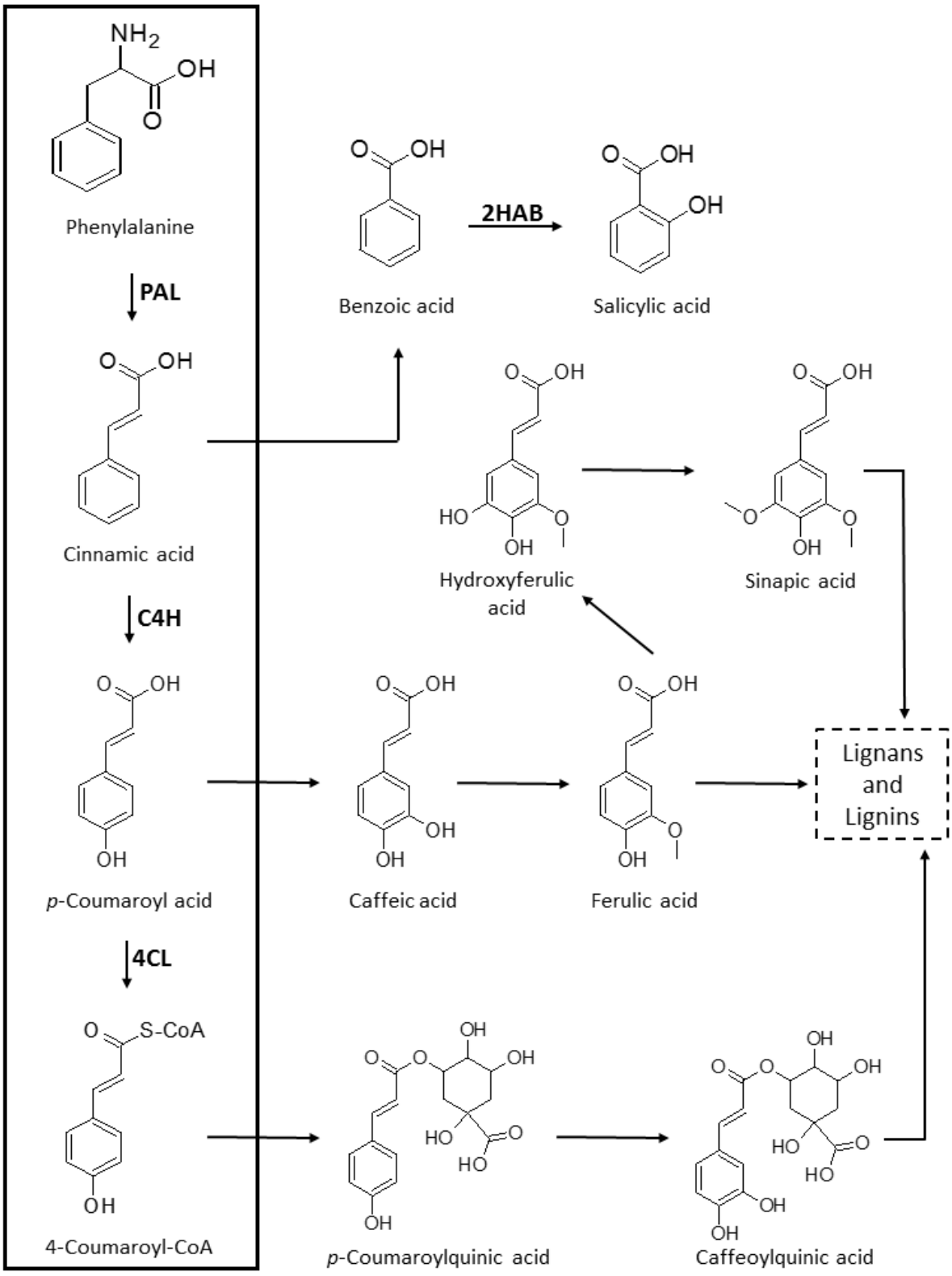


Figure I.4. Phenylpropanoids pathway (PAL: phenylalanine ammonia lyase; C4H: cinnamate-4-hydroxylase; 4CL: 4-coumaroyl-CoA ligase; 2HAB: 2-hydroxylase benzoic acid) (adapted from Vermerris and Nicholson, 2006).

This is further hydroxylated in the 4 position by cinnamate-4-hydroxylase (C4H) to form *p*-coumaric acid. The complex 4-coumaroyl-CoA ligase (C4L) catalyses the conversion of *p*-coumaric acid and coenzyme A to coumaroyl-CoA and three molecules of malonyl-CoA (Ferrer *et al.*, 2008). Alternatively, the cinnamic acid can also be metabolized to benzoic acid and then salicylic acid, the latter reaction being catalysed by the 2-hydroxylase benzoic acid (2HBA) (Vermerris and Nicholson, 2006).

The *p*-coumaric acid, through a number of hydroxylation and methylation reactions may also give rise to caffeic, ferulic, 5-hydroxyferulic and synaptic acids (Vermerris and Nicholson, 2006). The ferulic and synaptic acids, together with the *p*-coumaroyl-CoA are the precursors of lignans and lignins (Ferrer *et al.*, 2008).

The flavonoid biosynthesis is catalysed by the enzyme chalcone synthase (CHS), and consists in the condensation of thioesters of *p*-coumaroyl-CoA with three units of malonyl-CoA, forming a chalcone. This, by intramolecular cyclization gives a flavanone, naringenin, a reversible reaction catalysed by chalcone isomerase (CHI) (Martens and Mithöfer, 2005) (**Figure I.5**).

The oxidation of flavanones to di-hydroflavonols is catalysed by the enzyme flavanone 3-hydroxylase (F3H) while flavonol synthase (FLS) catalyses the oxidation of di-hydroflavonols to form flavonols (Vermerris and Nicholson, 2006).

The enzyme di-hydroflavonol reductase (DFR) catalyses the reduction of di-hydroflavonols to flavan-3,4-diols (leucoanthocyanidins), while the oxidation of *cis*-leucoanthocyanidin, catalysed by the enzyme leucoanthocyanidin oxidase (LDOX) or anthocyanidin synthase (ANS) gives way to anthocyanidins. These, in turn, are the precursor of anthocyanins by the action of 3-*O*-glycosyltransferases. The DFR is also involved in the formation of proanthocyanidins by the action of leucoanthocyanidin reductase (LAR), which catalyses the formation of catechins, that, by condensation, give rise to proanthocyanidins (Xie and Dixon, 2005).

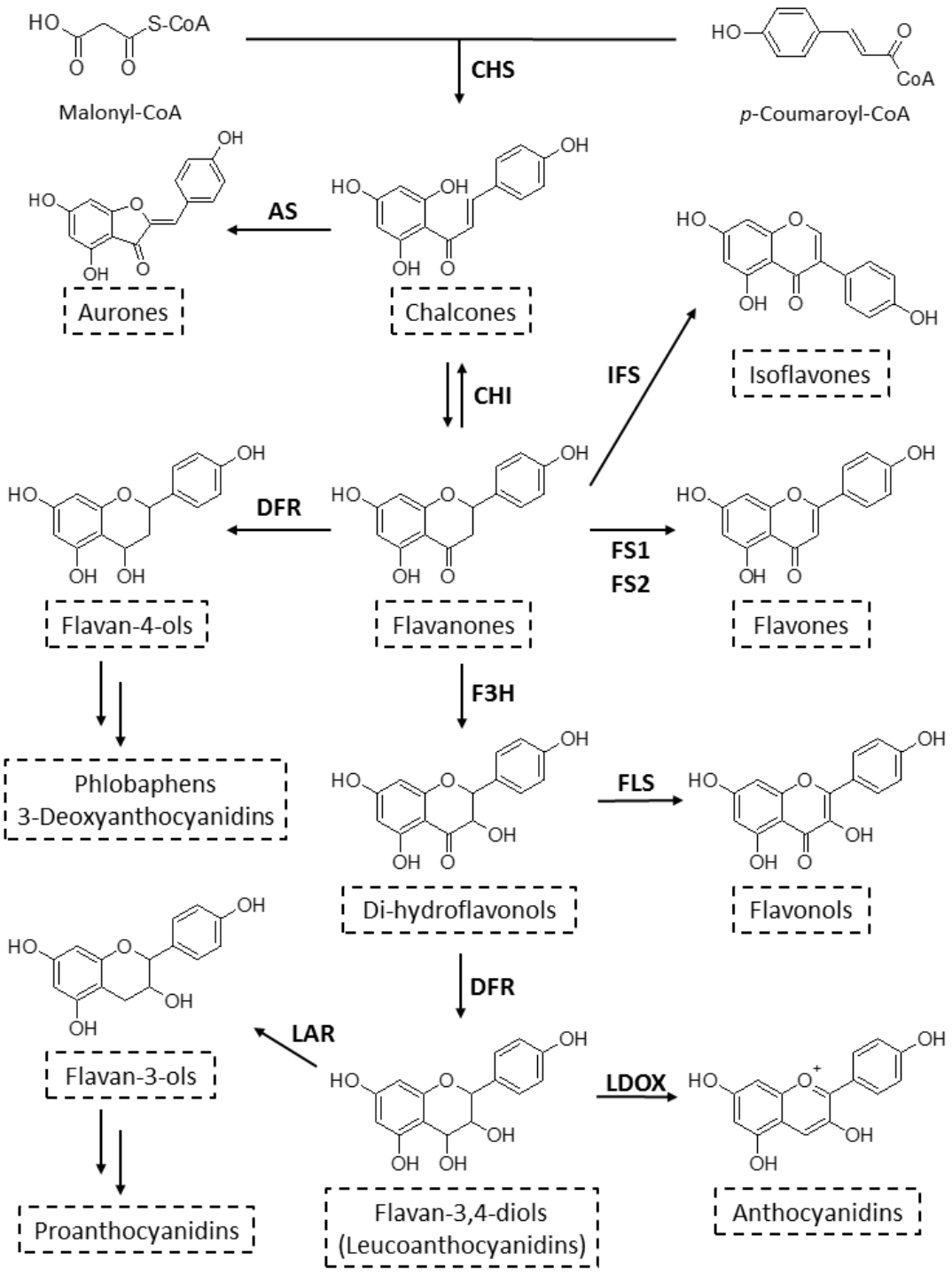


Figure I.5. Flavonoids biosynthesis (CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; FLS: flavonol synthase; LDOX: leucoanthocyanidin oxidase; FS1: flavone synthase 1; FS2: flavone synthase 2; DFR: di-hydroflavonol reductase; LAR: leucoanthocyanidin reductase) (adapted from Crozier *et al.*, 2006).

The synthesis of flavones is characterized by a great diversity of mechanisms. In one case, the flavone synthase 1 (FS1) removes two electrons of the carbons 2 and 3 of flavanone to form a flavone. Another possible mechanism depends on a mono-oxygenase, an enzyme from cytochrome P450, flavone synthase 2 (FS2), which catalyses the formation of an hypothetical 2-hydroxylflavanone, that is subsequently dehydrated to flavone (Martens and Mithöfer, 2005). The heme-oxygenase cytochrome P450 enzymes catalyse the oxidation of A and B rings and the unsaturation in the flavanones. The hydroxylation pattern of the B ring depends on the phenylpropanoid precursor and hydroxylases belonging to the cytochrome P450. The most common enzymes involved in hydroxylation of the B ring are flavone 3'-hydroxylase (F3'H), which leads to hydroxylation in the 3' carbon and flavone 3',5'-hydroxylase (F3'5'H) which results in hydroxylation at carbons 3' and 5'. These enzymes hydroxylate many flavonoids such as di-hydrokaempferol, kaempferol and apigenin (Ferrer *et al.*, 2008). Other frequent substitutions in flavonoids are the methylation catalysed by *O*-methyltransferases (*O*MTS) and glycosylations. The glycosylated forms of flavonoids are the most commonly found in nature. This type of substitution usually occurs through oxygen atoms incorporated in hydroxyl groups, but also through carbon atoms. Reactions are catalysed by glycosyltransferases (GTs) that use activated sugars with nucleotides as donor substrates. A wide variety of sugars can be combined: mono-, di-, tri-glycosides, either heterogeneous or homogeneous (Vogt and Jones, 2000). The acylations with aliphatic and aromatic acids, especially acetic, malonic, *p*-coumaric and ferulic also constitute one of the final steps in the biosynthesis, being catalysed by acyltransferases and requiring acyl-CoA as the acyl donor (Ferrer *et al.*, 2008).

2. Classification and natural sources

Phenolic compounds are a group of chemical substances, many of them present in, named phytochemicals, which comprise a large variety of structures. It is difficult to describe them chemically, 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 that they contain and to the structural elements binding these rings each other. The main classes of phenols are: phenol derivatives or simple phenols, phenolic acids, stilbenes, lignans, flavonoids, tannins, coumarins and anthraquinones (**Figure I.6**).

2.1 Simple phenols

Phenol derivatives are the most abundant phenols found in nature (**Figure I.7**). They are usually pyrogallol and phloroglucinol derivatives, but there are also di-hydroxylated derivatives, such as catechol, resorcinol and hydroquinone (Vermerris and Nicholson, 2006). The antioxidant activity of simple phenols depends on the position of the hydroxyl groups. Studies showed that the catechol group is the one that has greater activity, followed by hydroquinone and resorcinol, which in turn, is much greater to that of phenol (carbolic acid) (Kancheva, 2009).

Simple phenols also have anti-inflammatory activity, in particular by their action in the arachidonic acid cascade. The hydroxyl groups in *ortho* or *para* positions stimulate the synthesis of prostaglandin E_2 (PGE_2) and simultaneously inhibit the synthesis of leukotriene B_4 (LTB_4) (Alanko *et al.*, 1993).

2.2 Phenolic acids and derivatives

Characterized by a phenol ring and a carboxylic function, phenolic acids can be subdivided in two classes: benzoic acid derivatives and cinnamic acid derivatives (C_6-C_1 and C_6-C_3 skeletons, respectively) (**Figure I.8**). The benzoic acids, such as gallic acid and protocatechuic acid (PCA), are found in very few plants, like

blackberries, raspberries and tea (Shahidi and Naczk, 1996). Although the very low concentration of PCA in plants, Vitaglione and co-workers (2007) verified that the PCA concentration increases in the serum of human healthy volunteers with an anthocyanin-rich diet. So, PCA could be considered the major anthocyanin metabolite *in vivo*.

Hydroxycinnamic acids are *trans*-3-phenyl-2-propenoic acids differing in their ring substitution and that comprises 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 exists in artichoke, corn, wheat, rice and oat (Barnes *et al.*, 2007; Courts and Williamson, 2013). Ferulic acid is present in cereal grains and, in opposition to caffeic acid, is a less abundant phenolic acid (D'Archivio *et al.*, 2007).

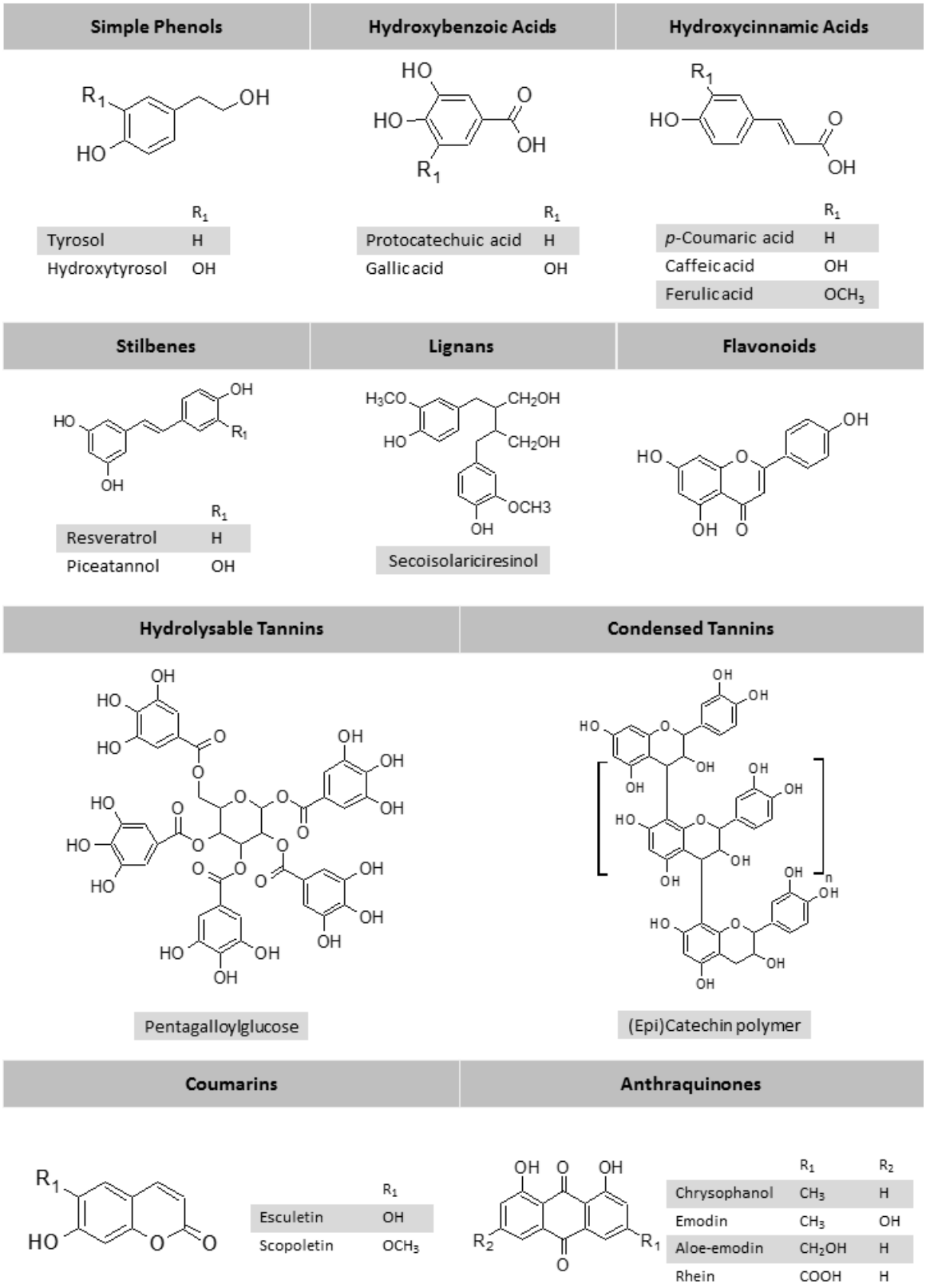


Figure I.6. Main classes of phenolic compounds.

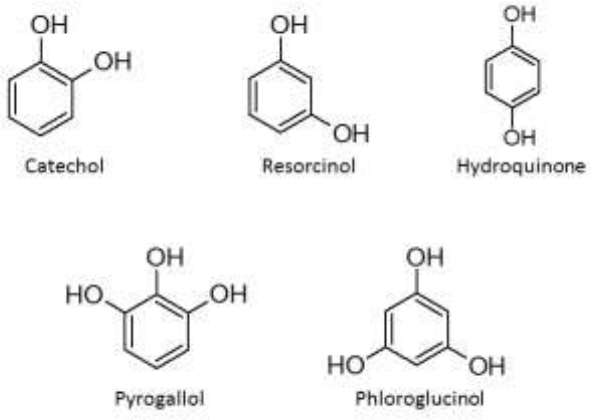


Figure I.7. Simple phenols (examples).

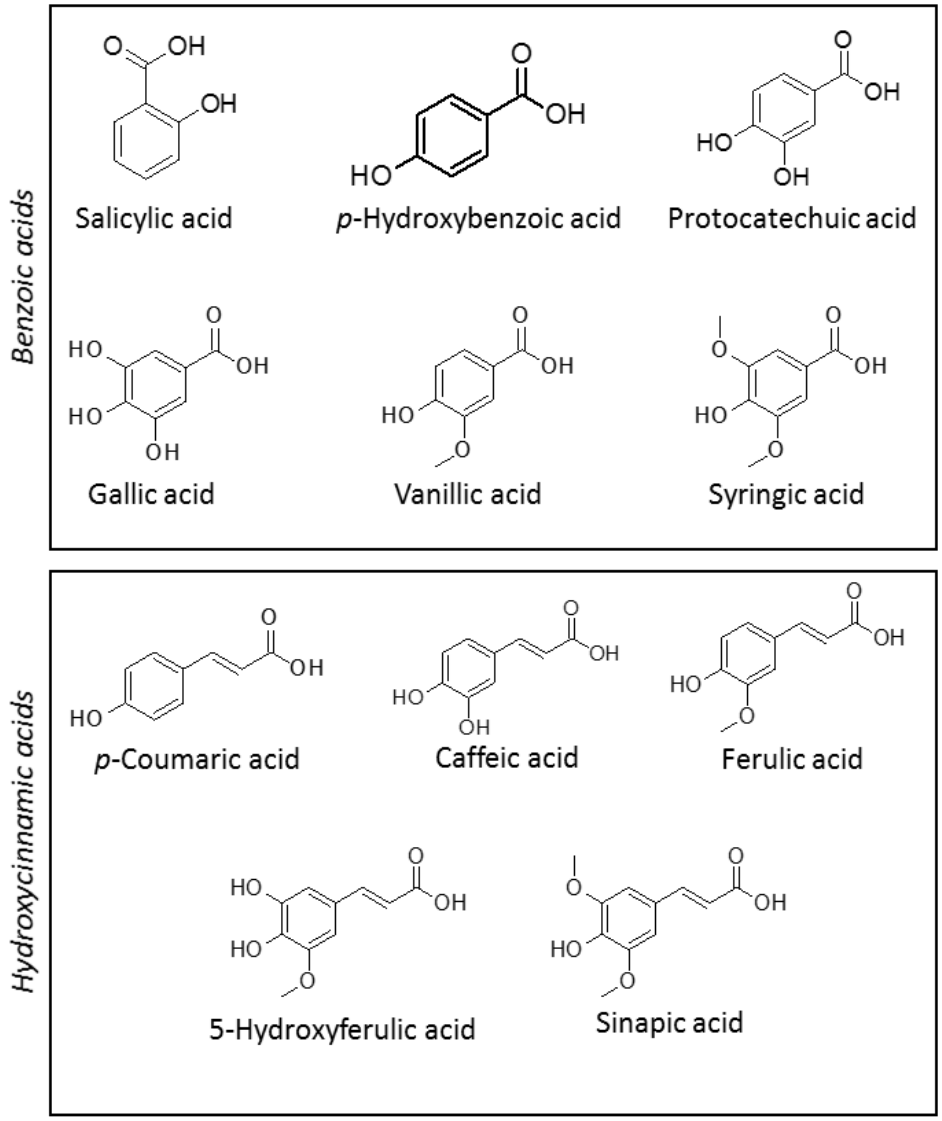


Figure I.8. Phenolic acids (examples).

2.3 Stilbenoids

Structurally, stilbenoids are hydrocarbons with a *trans*-ethene double bond substituted with a phenyl group on both carbon atoms of the double bond (**Figure I.9**). Stilbenes exist in low percentage in plants. Resveratrol and its isomer trans-resveratrol, which are the most abundant molecules of this class, are mainly found in grapes and wine (Zhang *et al.*, 2006), but also in *Reynoutria japonica* and *Polygonatum multiflorum* and in the heartwood of vascular plants such as *Cassia garrettiana* (Kimura *et al.*, 1995).

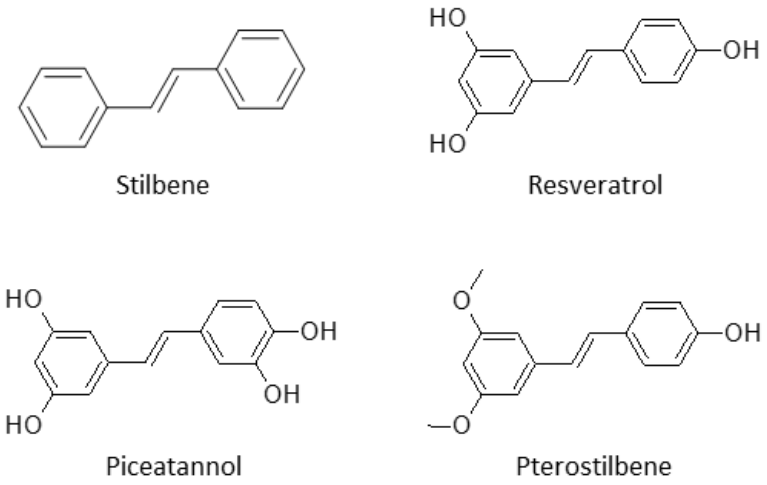


Figure I.9. Stilbenoids (examples).

2.4 Lignans

Lignans possess a dibenzylbutane skeleton derived from phenylalanine via dimerization of substituted cinnamic alcohols (**Figure I.10**). Lignans are mostly found in their free form and the main sources of these molecules are *Arctium lappa*, *Linum* spp., *Secale cereale*, *Triticum* spp., *Avena sativa* and *Hordeum vulgare* (Barnes *et al.*, 2007; D'Archivio *et al.*, 2007).

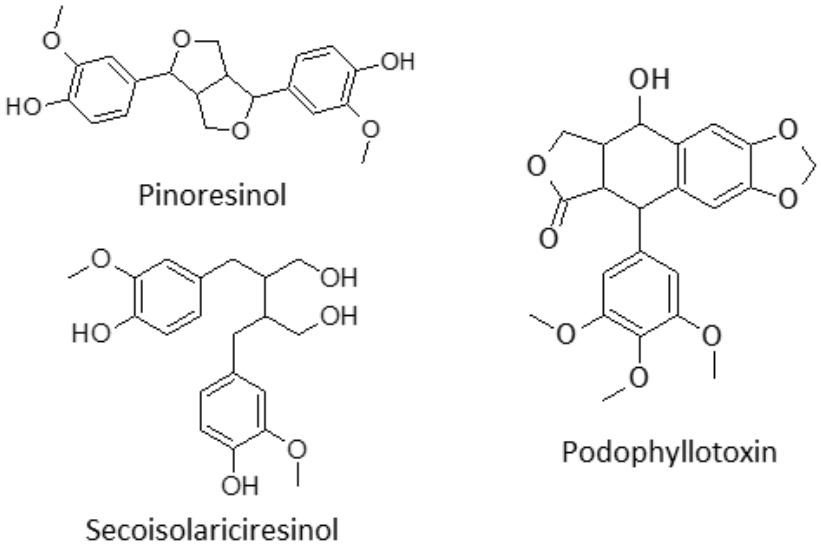


Figure I.10. Lignans (examples).

2.5 Flavonoids

Comprising more than 10,000 naturally occurring compounds, flavonoids are nearly ubiquitous in the kingdom of plants (in more than 9000 species) (Mouradov and Spangenberg, 2014). These molecules have a great structural diversity, but are all derived from a common biosynthetic pathway, the phenylpropanoid metabolic pathway, which incorporates precursors from both shikimic acid and acetate-malonate pathways, leading to the first flavonoid produced, the 2-hydroxychalcone. This basic compound serves as the central core for all other flavonoids, obtained by subsequent ring closure. The flavonoids derived from 2-hydroxychalcone are characterized by a common structure of 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). Considering the oxidation state and the functional group bonded to the C-ring, flavonoids are divided into subclasses (**Figure I.11**). Taking into account the oxidation state, the ring structure could be a heterocyclic pyran or a pyrone. The heterocyclic pyrans include flavans, flavan-3-ols, flavan-4-ols and flavan-3,4-diols which do not have unsaturated bonds on C-ring, or anthocyanidins, with 2 double bonds. The pyrones comprise a carbonyl residue at C4, with one double bond C2=C3 and a

hydroxyl group at C3 (flavonols), one double bond C2=C3 (flavones), without the double bond C2=C3 (flavanones), with one double bond C2=C3, but where the connection of the B-ring is through the C3 of C-ring instead of C2, as for other flavonoids (isoflavones), and the latter discovered neoflavonoids, which have some differences from the other C-ring pyrone flavonoids namely, the carbonyl group is on C2 instead of C4, the double bond is in C3=C4 and the connection of the B-ring is through the C4 of C-ring (Gomes *et al.*, 2008). Anthocyanidins like cyanidin, malvidin, delphinidin and pelargonidin are present in *Rubus* spp. *Vaccinium* spp. and *Symplocarpus foetidus* (Barnes *et al.*, 2007), being responsible for their remarkable colour. Flavonols are wide spread in vegetables, cereals, spices and herbs. Quercetin, myricetin and kaempferol represent flavonoids significantly found in foods. Flavones, like apigenin and luteolin, may be found in vegetables, but also in spices and herbs. Flavonols and flavones are also widely spread among medicinal plants such as *Hypericum spp.*, *Pneumus boldus*, *Eupatorium perfoliatum* and *Sambucus nigra* (Barnes *et al.*, 2007). The most frequent flavanones are naringenin and hesperidin, which can be extracted from citrus fruits and juices (Manach *et al.*, 2005). In general, flavonoids are also present in grassy plants, namely in grasses such as wheat, barley, rye, rice and sugarcane (Courts and Williamson, 2013). Finally, isoflavones are generally present in soy beans and other vegetables, being genistein and daidzein the most naturally occurring isoflavones.

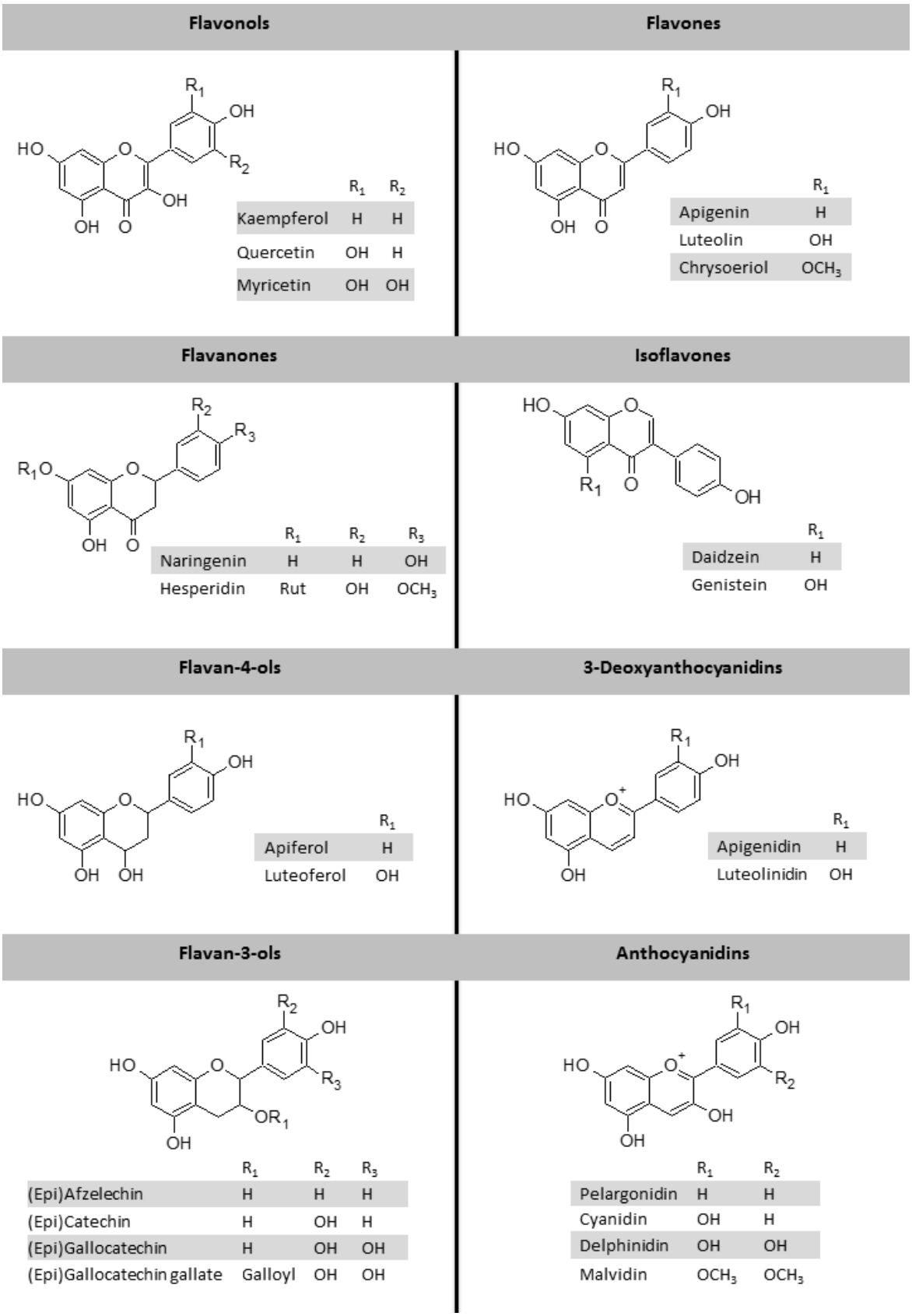
Within the various classes of flavonoids is common further differentiation, due to modifications of the flavonoids nucleus: additional or reduced hydroxylation; methylation of hydroxyl groups or of the aromatic core; isoprenylation of hydroxyl groups or of the flavonoids nucleus; methylenation of *ortho*-dihydroxyl 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 producing *C*-glycosides (Courts and Williamson, 2013).

C-glycosyl flavonoids are a less well studied sub-class of secondary plant metabolites in comparison to their more common and structurally diverse *O*-glycosyl relatives. The defining feature and divergent characteristic of their formation and subsequent nature is a *C*-*C* covalent bond between the aglycone flavonoid backbone and a sugar moiety: generally monomer glucose or galactose. This linkage is found in plant species, such as *C*-glycosyl flavonoids at either the 6 or 8 positions about the carbon backbone A-ring, and differs from *O*-glycosyl examples in the limited number of glycosylation positions, the form and range of attached saccharide polymerisation, and critically in the lack of an oxygen intermediate required for glycosidic linkage synthesis (Franz and Grün, 1983).

Another less common sub-class are the *O*-glycosyl-*C*-glycosyl flavones, characterised by *O*-glycosylation at the same positions as described for mono-*O*-glycosyl flavonoids in addition to *C*-glycosylation elsewhere on the flavone backbone, or *O*-glycosylation at one of the hydroxyl groups of a *C*-linked sugar moiety. The latter form of *O*-glycosylation occurs most usually at the 2 or less frequently at the 6 carbon of a *C*-linked hexose sugar (Ferreres *et al.*, 2007). The significance of the *C*-glycosidic linkage to the overall flavonoid structure is higher than might be imagined at first in view of the seemingly relatively minor chemical divergence from the *O*-glycosidic linkage.

The *C*-glycosidic bond between a sugar moiety and flavonoid carbon skeleton largely protects the flavonoid glycoside from the hydrolytic effect of both acidic and non-bacterial enzymatic treatments known to readily cleave *O*-glycosidic linkages, leading to fundamental differences in the analysis, degradation, pharmacokinetics and bioactivity of those flavonoids possessing a *C*-glycosyl group (Harborne, 1965).

Most flavonoids do not occur in plants as aglycones, flavonoids *O*-glycosides being the most abundant. The exceptions are the flavan-3-ols like catechin, epicatechin and epigallocatechin gallate present in fruits, tea, red wine and cocoa products.



	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Myricetin	OH	OH

	R_1
Apigenin	H
Luteolin	OH
Chrysoeriol	OCH_3

	R_1	R_2	R_3
Naringenin	H	H	OH
Hesperidin	Rut	OH	OCH_3

	R_1
Daidzein	H
Genistein	OH

	R_1
Apiferol	H
Luteoferol	OH

	R_1
Apigenidin	H
Luteolinidin	OH

	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

Figure I.11. Flavonoids (main classes and examples).

2.6 Tannins

Tannins are a group of water-soluble polyphenols with high molecular weights (500 to 3000 Da) (**Figure I.12**). This class of phenolic compounds is mainly constituted by hydrolysable and condensed tannins, with structural units corresponding to phenolic acids or flavans, respectively.

Hydrolysable tannins are formed by more or less complex conjugations of gallic acid (gallotannins) or ellagic acid (ellagitannins) or both. On the other hand, condensed tannins, also called proanthocyanidins, are built with flavanic units. They may be classified according to different criteria: the nature of the basic unit or the type of interflavanic linkage. The flavans that may constitute the condensed tannins are diverse: simple flavans (hydroxyl group is absent in the C-ring), flavan-3-ols, flavan-4-ols and flavan-3,4-diols. In what concerns the interflavanic bond, proanthocyanidins may be grouped as A-, or B-type. B-type proanthocyanidins are characterized by a singly linked flavanyl units, usually between C4 of the chain-extension unit and C6 or C8 of the chain-terminating moiety. In contrast, proanthocyanidins of the A-type possess an unusual second ether linkage to C2 of the terminating-unit. This feature introduces a high degree of conformational stability at the interflavanyl bonding axes of dimeric analogues.

Mostly found in complexes with alkaloids, polysaccharides and proteins, tannins are generally present in plant barks and also in rhubarb (*Rheum rhabarbarum*) and cereals (Rangkadilok *et al.*, 2007).

2.7 Coumarins

Coumarins are lactones obtained by cyclization of hydroxycinnamic acids, belonging to the phenolic compounds group with the basic skeleton of C_6-C_3 (**Figure I.13**). Coumarins are present in plants in free and glycosylated forms.

In general, coumarins are characterized by high chemical diversity, mainly differing in the degree of oxygenation of their benzopyrone 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). Herbaceous plants, tonka beans (*Dipteryx odorata*), vanilla grass (*Hierochloe odorata*) and sweet woodruff (*Galium odoratum*) are some sources of coumarins (Huang *et al.*, 2010).

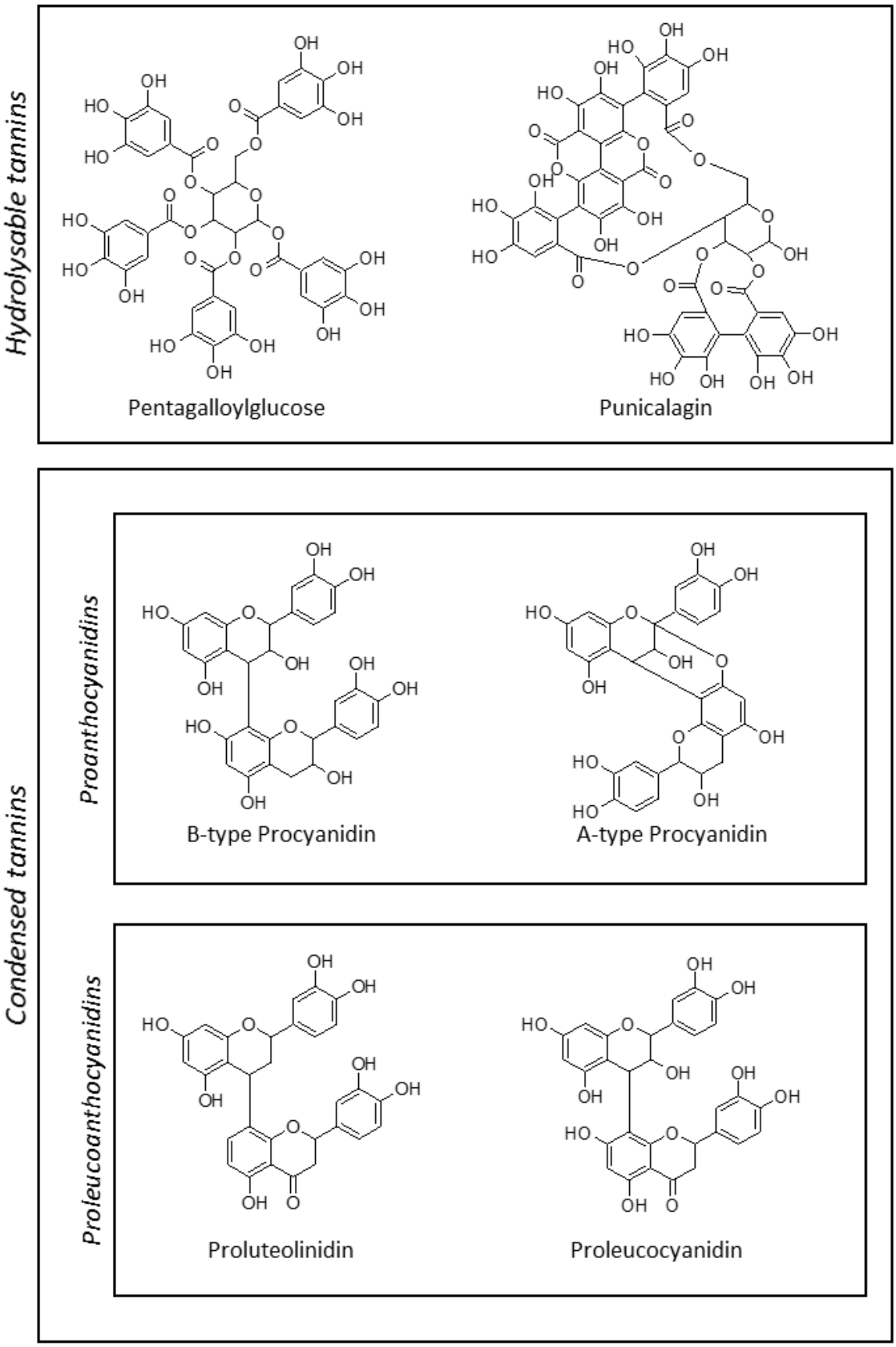


Figure I.12. Tannins (main classes and examples).

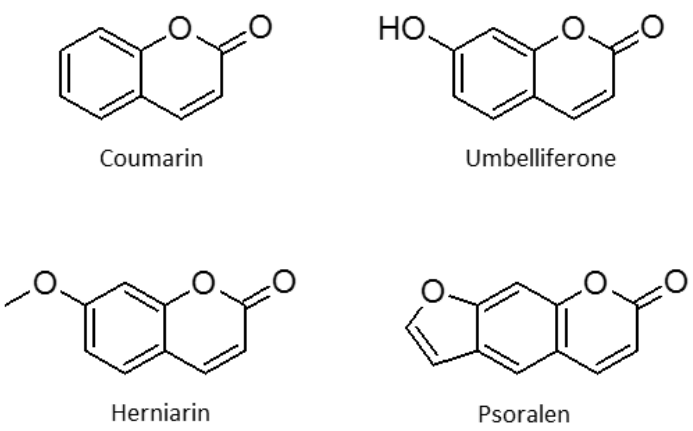


Figure I.13. Coumarins (examples).

2.8 Anthraquinones

Also known as hydroxyanthracenic compounds, anthraquinones are a group of colourful 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 (**Figure I.14**) (Vermerris and Nicholson, 2006). The reduced forms of anthraquinones (anthrones) may also dimerize to form dianthrones and these dimmers 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 (Vermerris and Nicholson, 2006).

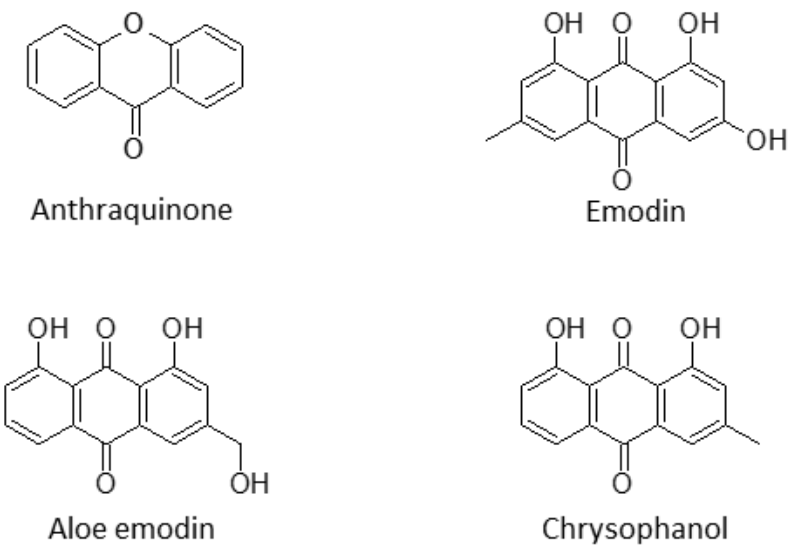


Figure I.14. Anthraquinones (examples).

3. *In vivo* anti-inflammatory properties

A lot of *in vitro* studies evidence the anti-inflammatory properties and mechanisms of action of phenolic compounds, however less *in vivo* studies have been published (Gonzalez-Paramas *et al.*, 2011). In this review, some of the most representative compounds are referred (**Table I.5**).

Different animal models and different routes of administration have been used for *in vivo* studies; consequently it is difficult to establish structure-activity relationships. Nonetheless, some studies have shown that C2-C3 double bound is essential for *in vivo* activity of flavonoids. Additionally, their potency is also related to the pattern and number of hydroxylation(s) on the A/B-ring (Pelzer *et al.*, 1998), 5, 7-hydroxylation on the A-ring and 3',4'-di-hydroxylation on the B-ring, which is the most favourable (Gonzales *et al.*, 2015).

Table I.5. *In vivo* anti-inflammatory properties of phenolic compounds (cont.).

Phenolic Compounds	Anti-inflammatory activity*	*In vivo* model	Route of administration	Reference
Phenolic Acids and Derivatives				
Verbascoside	Colon damage, mediated by PPARα	Dinitrobenzene sulfonic acid-induced experimental colitis	Oral	Esposito *et al.*, 2010
Curcumin	Ear oedema	TPA-induced ear oedema in mice	-	Huang *et al.*, 1991
	NF-κB binding activity	Streptozotocin-induced diabetic rats	Oral	Kowluru and Kanwar, 2007
	PPAR-γ *is activated* by induction of protein expression	TNBS-induced colitis in rat	Intraperitoneal injection	Zhang *et al.*, 2006
	Colitis symptoms	TNBS-induced colitis in mice	Oral	Camacho-Barquero *et al.*, 2007
	p38 MAPK activation			
	NO production and iNOS expression			
	iNOS mRNA levels in liver	LPS-injected mice	Oral	Chan, 1995
	COX-2 expression	TNBS-induced colitis in mice	Oral	Camacho-Barquero *et al.*, 2007
	TNF-α levels			
	IL-1β levels	Streptozotocin-induced diabetic rats	Oral	Kowluru and Kanwar, 2007
	Inflammatory responses in pancreatic beta-cells	Streptozotocin-induced diabetic rats	Intragastric	Rashid and Sil, 2015
	Activates Nrf2 NF-κB pathway	Male C57BL/6 mice	Intragastric	Zeng *et al.*, 2015
Stilbenes				
Resveratrol	Endotoxemia symptoms	LPS-injected mice	Intraperitoneal injection	Sebai *et al.*, 2009
	Plasma NO			
	Colonic injury	TNBS-induced colitis in rats	Oral	Martín *et al.*, 2004
	COX-2 expression and PGD_2 levels			
	IL-1β levels			
	Atherosclerotic lesions ICAM-1 and VCAM-1 expression	Apo E-deficient (apo $E^{-/-}$) mice (Atherosclerosis model)	Oral	Do *et al.*, 2008
	Inflammasome activation	Hypercholesterolemic rats	Oral	Deng *et al.*, 2015

Table I.5. *In vivo* anti-inflammatory properties of phenolic compounds (cont.).

Phenolic Compounds	Anti-inflammatory activity*	*In vivo* model	Route of administration	Reference
Flavonoids				
Apigenin	Serum IgE levels Serum IL-4 levels	Ovalbumin-sensitized mice	Oral	Yano *et al.*, 2007
	Oxidative damage inflammatory responses	Male CBA/CaJ mice	Intraperitoneal injection	Rithidech *et al.*, 2012
	IL-6, IL-8, and ICAM-1 expression	Female BALB/c mice	Intraperitoneal injection	Wang *et al.*, 2012
	Severity of experimental autoimmune encephalomyelitis	C57BL/6 and SJL/J mice	Intraperitoneal injection	Ginwala *et al.*, 2015
Luteolin	Lethal endotoxin shock Leukocyte infiltration in the liver Serum TNF-α levels ICAM-1 expression in the liver	LPS-induced lethal toxicity in mice	Intraperitoneal injection	Kotanidou *et al.*, 2002
	Alveolar wall Thickness Oedema Inflammatory cell infiltrates Blood vessels damage	LPS-induced mouse endotoxin shock model	Intraperitoneal injection	Chen *et al.*, 2014
	TNF-α-induced vascular inflammation	C57BL/6 mice	Intraperitoneal injection	Jia *et al.*, 2015
	Clinical symptoms in DSS-induced colitis	Female C57BL/6 CrSlc mice	Intragastric	Nishitani *et al.*, 2013
Isoorientin	MPO activity	Carrageenan-induced mice with pleurisy	Intraperitoneal injection	Zucolotto *et al.*, 2009
	Hyperpermeability, expression of CAMs, and adhesion and migration of leukocytes	Male C57BL/6 mice	Intravenous injection	Lee *et al.*, 2014
Chrysin	Serum IgE levels Serum IL-4 levels	Ovalbumin-sensitized mice	Oral	Yano *et al.*, 2007
Wogonin	NO levels in serum	LPS-treated Balb/c mice	Intravenous injection	Shen *et al.*, 2002
Wogonin	COX-2 protein expression and PGE_2 production Neutrophil infiltration	TPA-treated mice	Topical application in dorsal skin	Park *et al.*, 2001
Genistein	Inflammation and joint destruction (histological analysis)	Collagen induced arthritis in mice	Subcutaneous injection	Verdrengh *et al.*, 2003

Table I.5. *In vivo* anti-inflammatory properties of phenolic compounds (cont.).

Phenolic Compounds	Anti-inflammatory activity*	*In vivo* model	Route of administration	Reference
Quercetin	Lethal endotoxic shock	LPS or LPS plus D-galactosamine treated mice	Intraperitoneal injection	Takahashi *et al.*, 2001
	Severity of adjuvant-induced arthritis	Chronic rat adjuvant-induced arthritis	Oral or intra-cutaneous injection	Mamani-Matsuda *et al.*, 2006
	Eosinophil number	Ovalbumin-sensitized mice	Oral	Rogerio *et al.*, 2007
	NO levels in serum	LPS-treated Balb/c mice	Intravenous injection	Shen *et al.*, 2002
	COX-2 mRNA and PGE_2 production	Carrageenan-injected rats	-	Morikawa *et al.*, 2003
	TNF-α, RANTES, MIP-2 protein levels			
Isoquercetin	COX-2 mRNA levels and PGE2 production	Carrageenan-injected rats	-	Morikawa *et al.*, 2003
	Cytokine production, namely TNF-α			
	Chemokine production: CXCL2 and CCL5			
Kaempferol	Tyk2-STAT1/3 signaling modulation	Asthmatic mice	Oral	Gong *et al.*, 2013
	Src kinase activity UVB-induced COX-2 expression	Female ICR mice	Topic	Lee *et al.*, 2010
	Acute inflammatory and symptoms in gastritis and pancreatitis	Male ICR and Balb/c mice	Oral	Kim *et al.*, 2015
Rutin	Lethal endotoxic shock	LPS or LPS plus *D*-galactosamine treated mice	Intraperitoneal injection	Takahashi *et al.*, 2001
	Serum TNF-α levels			
	NO levels in serum	LPS-treated Balb/c mice	Intravenous injection	Shen *et al.*, 2002
Morin	Colonic damage	TNBS-induced colitis in mice	Oral	Galvez *et al.*, 2001
	NOS activity			
	LTB_4 levels			
	IL-1β levels			
Icariin	Paw oedema	Carrageenan-induced paw oedema	Oral	Liu *et al.*, 2010

Table I.5. *In vivo* anti-inflammatory properties of phenolic compounds.

Phenolic Compounds	Anti-inflammatory activity*	*In vivo* model	Route of administration	Reference
Epigallocatechin gallate	Polymorphonuclear leukocytes infiltration	TPA-treated mice	Topical application in dorsal skin	Wei and Frenkel, 1993
	Arthritis histological scores and clinical symptoms	Mice injected with monoclonal antibodies against collagen (arthritis model)	Intraperitoneal injection	Morinobu *et al.*, 2008
	Colitis symptoms	TNBS-induced colitis in mice	Intraperitoneal injection	Abboud *et al.*, 2008
	NF-κB activation through: IκB phosphorylation and degradation p65 translocation to nucleus DNA binding	TPA-treated mouse Ischemic-reperfused rat myocardium	Oral and intravenous injection	Aneja *et al.*, 2004; Kundu and Surh, 2007
	AP-1 activity through: c-Jun phosphorylation DNA binding	TNBS-induced colitis in mice Ischemic-reperfused rat myocardium	Intraperitoneal and intravenous injection	Abboud *et al.*, 2008; Aneja *et al.*, 2004
	Plasma IL-6 levels	Ischemic-reperfused rat myocardium	Intravenous injection	Aneja *et al.*, 2004
	Pro-inflammatory responses in peripheral leukocytes	Male GK and Wistar rats	Oral	Uchiyama *et al.*, 2013
Chrysophanol	NF-κB p65 activation COX-2 protein expression IL-6 production	Dextran sulfate sodium-induced colitis in mice	Oral	Kim *et al.*, 2010

*inhibition of indicated events, unless where it is referred.

Structure-activity relationships constitute 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. In **Table I.6** it is described the correlation between anti-inflammatory properties of flavonoids and the presence of specific functional groups. Flavonoids possess anti-inflammatory action in part due to their intrinsic antioxidant capacity. The scavenging of oxidizing species by flavonoids is related to the presence of the double bond between carbons 2 and 3 in the C-ring of the flavonoid skeleton (Bonfili *et al.*, 2008). The inhibition of nitric oxide (NO) production and enzymes involved in the production of prostaglandins (PGs) and leukotrienes (LTs), like phospholipase A2 (PLA2), 5- and 12-lipooxygenases (LOXs), is also dependent on double bond between C2 and C3 (Kim *et al.*, 2004). It was demonstrated that flavones with this double bond were more potent than homologous flavanones, which have a single bond between C2 and C3 (Kim *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 tumor necrosis factor (TNF)-α-induced intercellular adhesion molecule (ICAM)-1 expression by the luteolin (Benavente-García and Castillo, 2008).

The hydroxylation of A-ring of flavonoids, in particular 5- and 7-hydroxylation(s) is important in antioxidant capacity (Bonfili *et al.*, 2008), inhibition of NO production (Kim *et al.*, 1999, 2004) and cell adhesion molecules expression, such as ICAM-1 (Gerritsen *et al.*, 1995; Suh *et al.*, 2006). Furthermore, the presence of hydroxyl group at position 3 of the C-ring slightly blocks the inhibition of ICAM-1 expression by flavonoids (Suh *et al.*, 2006). 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). Additionally, the presence of 8-methoxyl group in the A-ring affected the inhibition of NO production favorably (Kim *et al.*, 1999).

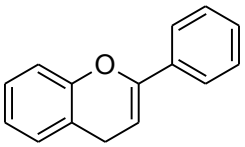
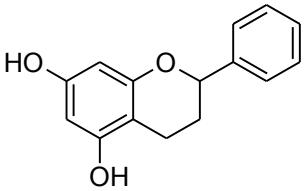
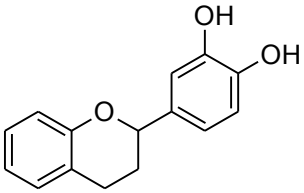
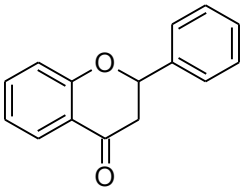
The flavonoids bioavailability is correlated with their structure. In fact, the number of hydroxyl groups affects the affinity of flavonoids for the cellular membranes, influencing their structure, fluidity, permeability and the intestinal

absorption (Bonfili *et al.*, 2008). The potency of anti-inflammatory activity of flavonoids *in vivo* depends on the patterns and number of hydroxyl groups on the B-ring. For example, 3',4'-dihydroxyl (catechol type) or 3',4'-hydroxyl/methoxyl (guaiacol type) groups were important for inhibiting granulomatous inflammation (Kim *et al.*, 2004). The 4´-OH substitution in the B-ring increases the potency of flavonoids to inhibit lipopolysaccharide (LPS)-induced NO production and is important in ICAM-1 expression inhibitory properties of flavonoids (Benavente-García and Castillo, 2008). Finally, TNF-α inhibitory activity is promoted by the existence of hydroxyl group in the C3' and C4' (B-ring), and the absence of these groups may reverse the flavonoids action (Ueda *et al.*, 2004). The presence of a carbonyl function at position 4 is required for the optimal inhibition of TNF-α-induced ICAM-1 expression, such as it was shown for luteolin (Benavente-García and Castillo, 2008).

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

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

Table I.6. Anti-inflammatory structure-activity relationships of flavonoids.

Key functional group	Structure	Anti-inflammatory activity*	Reference
C2-C3 double bond		NO production PLA_2 activity 5- and 12-LOXs activity ICAM-1 expression	Benavente-García and Castillo, 2008; Bonfili *et al.*, 2008; Kim *et al.*, 2004
5,7-dihydroxyl		NO production TNF-α production ICAM-1 expression	Bonfili *et al.*, 2008; Chen *et al.*, 2004; Gerritsen *et al.*, 1995; Kim *et al.*, 1999, 2004; Ueda *et al.*, 2004
3',4'-dihydroxyl		Proteasome activity Granulomatous inflammation TNF-α production ICAM-1 expression	Benavente-García and Castillo, 2008; Bonfili *et al.*, 2008; Kim *et al.*, 2004; Shie *et al.*, 2010; Ueda *et al.*, 2004
4-carbonyl		Proteasome activity ICAM-1 expression	Benavente-García and Castillo, 2008; Chen *et al.*, 2005

*inhibition of indicated enzymes and/or inflammatory mediators.

4. *In vivo* bioavailability

Phenolic compounds are currently receiving much attention due to their beneficial health effects related to their antioxidant (Xiao *et al.*, 2013), anti-inflammatory (Nichols and Katiyar, 2010), cardioprotective (Khurana *et al.*, 2013), cancer chemopreventive (Andrae-Marobela *et al.*, 2013), and neuroprotective properties (Albarracin *et al.*, 2012). Consequently, polyphenols can be considered healthy compounds, and claim to have health-promoting or

disease-preventing properties.

Significant advances have been achieved during the last decade, concerning the metabolism of polyphenols *in vitro*, but scarce data has been presented about what really happens in a completely different environment: the human body (Tarko *et al.*, 2013). A purposed scheme for digestive metabolism of polyphenols in humans is depicted in **Figure I.15.**

Bioavailability differs greatly from one polyphenol to another, so that the most abundant polyphenols are not necessarily those leading to the highest concentrations of active metabolites in target tissues. Knowledge of the bioavailability and metabolism of the various polyphenols is necessary to evaluate their biological activity within target tissues (Maqueda, 2012). However, most of the *in vitro* bioactivities of polyphenols remain uncharted *in vivo* (Li and Hagerman, 2013). Notwithstanding, some studies have reported the pharmacokinetics and tissue distribution of phenolics in liver, kidney, lung and heart (Wenzel and Somoza, 2005), but the plasma pharmacokinetics and tissue distribution of polyphenols and their metabolites have not yet been accounted in a satisfying manner (Lin *et al.*, 2012). Although *in vivo* studies have been performed to verify the biological effects of polyphenols, reports of its bioavailability, pharmacokinetics and biological fate are few. Also, data indicating the circulating concentration, as well as their metabolites after oral administration is needed (Coni *et al.*, 2000).

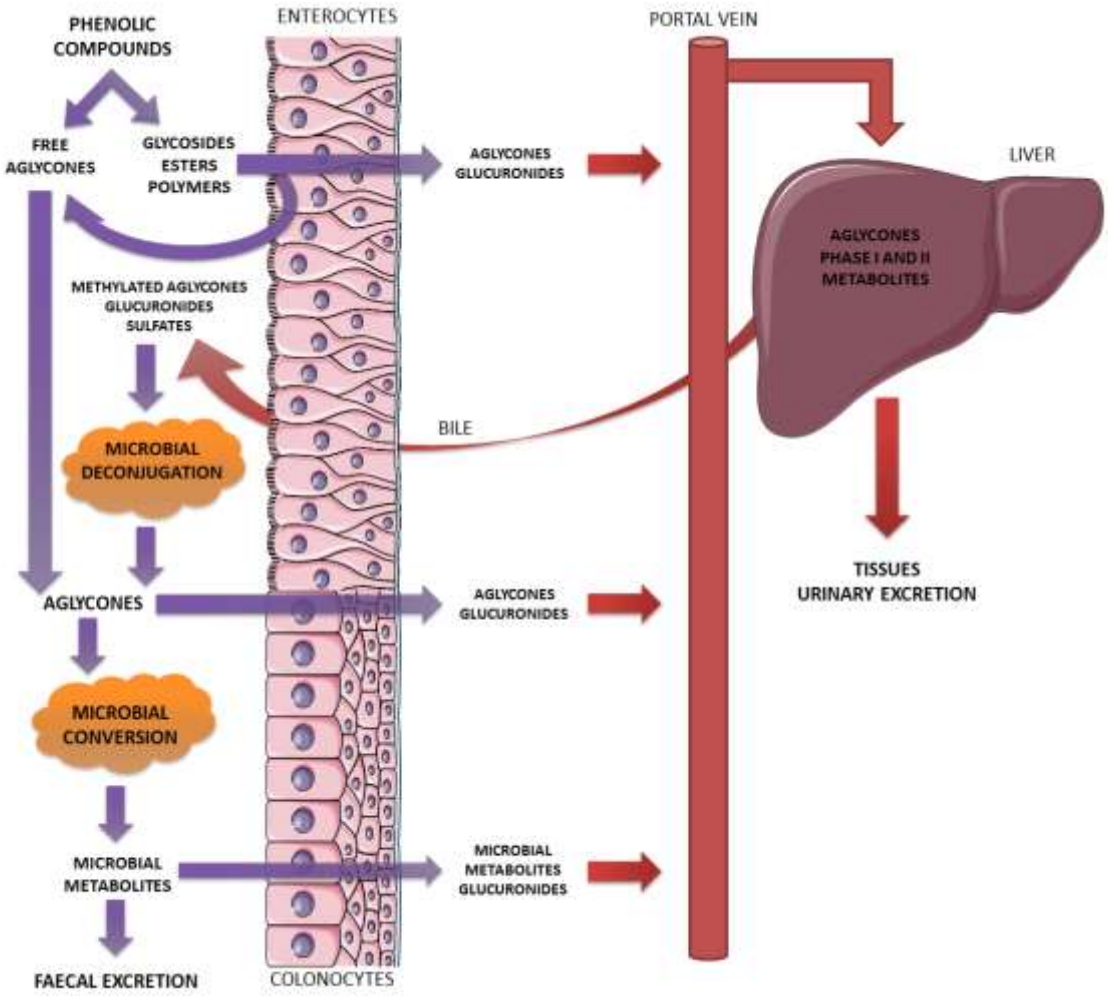


Figure I.15. Metabolism of polyphenols in human digestive system.

The kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol provided as pure compound, plant extract or whole food/beverage have been investigated (Manach *et al.*, 2005). Such knowledge will allow evaluation of polyphenol intake and enable epidemiologic analysis that will in turn provide an understanding of the relation between the intake of these substances and the risk of development of several diseases. Furthermore, not all polyphenols are absorbed with equal efficacy. Some exhibit very low bioavailability, e.g. resveratrol (Santos *et al.*, 2011), others show high plasmatic peaks, and a significant amount of polyphenols is converted in a wide range of metabolites. Polyphenols are metabolized by intestinal and hepatic enzymes and by the intestinal microflora (Manach *et al.*, 2004). Many studies have focused on

the bioactivity of one specific molecule in aglycone form, frequently administrated at supraphysiological doses, whereas the plant extracts and foods contain complex and often poorly characterized mixtures with multiple additive or interfering activities (Steiner *et al.*, 2008). Furthermore, recent human studies indicate that plasma concentrations and urinary excretions of gut microbial metabolites can exceed those of host origin, especially for polyphenols that are not easily absorbed in the upper gastrointestinal tract and persist untill the colon (van Dorsten *et al.*, 2010).

Flavonoid glycosides are a particularly relevant group of polyphenols since they represent the majority of the bioactive constituents ingested by humans. Deglycosylation has often been referred to as a critical step in the "activation" of flavonoids following human consumption (Hollman and Katan, 1998; Walle *et al.*, 2005). This appears to be generally true for *O*-glycosylated compounds, because buccal, intestinal or colonic enzymatic hydrolysis results in improved bioavailability of the resulting aglycone (Hollman and Katan, 1998; Walle *et al.*, 2005). However, greater resistance of the *C*-glycosidic linkage to hydrolysis dictates that the bioavailability of *C*-linked flavonoid glycosides is somewhat different. Increasing evidence suggests that deglycosylation is not a prerequisite for *C*-glycosyl flavonoid absorption in the small intestine, resulting in the presence of the intact glycosyl flavonoid in human urine following oral consumption (Courts and Williamson, 2013). Generally, *O*-glycosylated structures are not detected *in vivo* due to first-pass intestinal and hepatic hydrolysis, presumably catalysed by broad-specificity of the *β*-glucosidases (Day and Williamson, 2007). *C*-Glycosyl flavonoids derivatives may survive to hepatic hydrolysis for reasons outlined previously, explaining their seemingly unusual presence in human urine, after oral ingesting (Courts and Williamson, 2009; Hasslauer *et al.*, 2010). As such, it is very likely that *C*-glycosylation does not confer diffusive flavonoid absorption *per se*, rather that the *C*-glycosyl bond of these compounds has greater stability *in vivo*. While the *C*-glycosidic linkage appears to remain intact in the upper- and mid-gastrointestinal tract, and no mammalian *C*-deglycosylating enzyme known with specificity toward flavonoid structures exists, increasing evidence suggests that

bacteria capable of cleaving the *C*-glycosidic linkage (putatively via expression of *C*-deglycosylating enzymes) are present in the colon, as outlined in **Figure I.16** (Courts and Williamson, 2013).

Advances in the understanding of phenolic metabolism have been made possible by improvements in the analytical methods used, particularly the use of reverse phase HPLC-MS. Whereas most studies up to the late 1990s measured total aglycones in plasma and urine, after chemical or enzymatic deconjugation or both. Several recent works report the polyphenol conjugate composition of plasma, urine, faeces and/or tissues, after the administration of pure polyphenols or polyphenol-rich foods and beverages (Kroon *et al.*, 2004). Analysing biological samples in the context of bioavailability and pharmacokinetic studies with natural compounds, one must be aware that extensive metabolism and biotransformation occurs. Different from other chemically active pharmaceutical compounds, polyphenols are often subject to metabolism by the intestinal microflora prior to absorption. Assessment of unknown metabolites might be achieved by monitoring all analytes, which show the plasma concentration time kinetics in studies for single dose (Bhattaram *et al.*, 2002). The identification of metabolites is a challenge and requires more complex studies taking into account many possible influencing factors, such as age, gender, inter-individual bioavailability variation and also methodological approaches (Azorín-Ortuño *et al.*, 2010; Maqueda, 2012).

Studies of the mechanisms of the biological effects of polyphenols on human cell lines should use the metabolites produced *in vivo* (conjugates of the original aglycones or gut microbiota metabolites, such as methyl ethers, glucuronides and sulfates) and at the concentrations reached in biological fluids and tissues (nanomolar range) to provide real biological significance (Tomás-Barberán and Andrés-Lacueva, 2012).

Animal models, as close to humans as monkeys and as simple as the worm *Caenorhabditis elegans*, have been successfully used in polyphenols and health research. These have been particularly useful in toxicological and in tissue distribution studies, which are essential to understand the metabolites that can be active *in vivo*. The model of *C. elegans* provides a smart methodology for testing

the activity of polyphenols in a simple and repetitive manner that can be very useful for screening studies (González-Manzano *et al.*, 2012).

Most published studies are based on animal models, with the majority using rodents and primates. Although polyphenol metabolism has been described in humans and several other species, little attention has been given to the interspecies differences, which could be crucial in explaining discrepancies in physiologic effects of phenols in different animal models compared with humans (Gu *et al.*, 2006). Apart from the inherent differences between species, there are many factors that may influence in polyphenols' different metabolic profiles among studies, as like administration procedure, sample preparation (Wenzel *et al.*, 2005), extraction method (Wenzel *et al.*, 2005), polyphenols vehicles (Azorín-Ortuño *et al.*, 2010), dosage (Azorín-Ortuño *et al.*, 2010), animal gender (Cassidy *et al.*, 2006), polymorphisms (Miners *et al.*, 2002), circadian variation of enterohepatic circulation (Almeida *et al.*, 2009), anesthetic procedure, as well as the analytical procedure.

The anesthetic procedure is important, namely when animal and human studies are compared, because animal handling requires anesthesia commonly, contrary to studies in humans. Anesthetic drugs can affect important aspects of metabolism, such as gastric emptying, induction and or/inhibition of phase I and phase II enzymes, intestinal motility and inhibition of intestinal transporters (BCRP and MRP) (Azorín-Ortuño *et al.*, 2010).

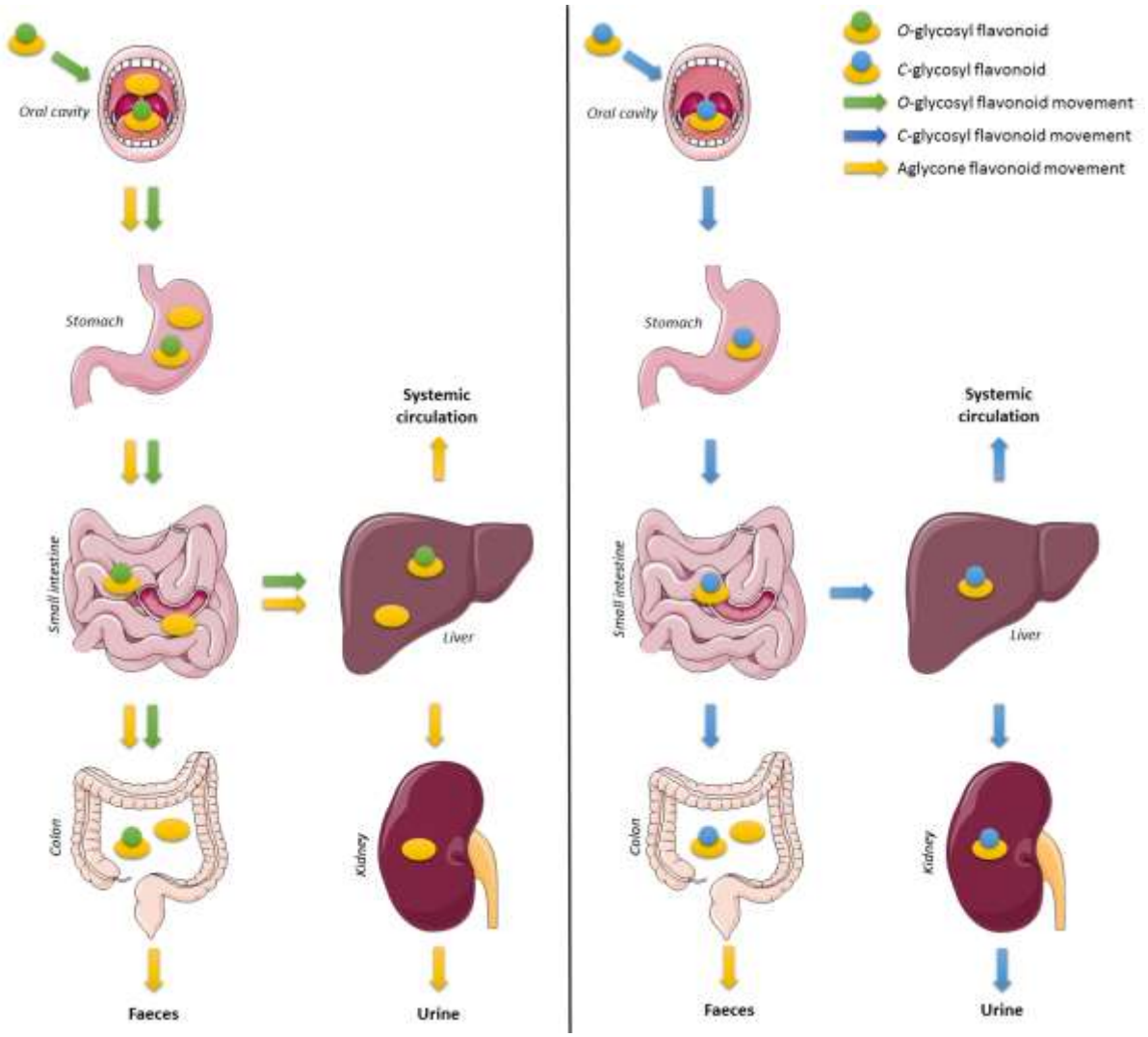


Figure I.16. Metabolism of *O*- and *C*-glycosyl flavonoids in humans.

4.1 Pharmacokinetics

Pharmacokinetics is a fundamental scientific discipline that underpins applied therapeutics. It provides a mathematical basis to assess the time course of drugs and their effects in the body. It enables to quantify the following processes: Absorption, distribution, metabolism and excretion (ADME). A variety of techniques is available for representing the pharmacokinetics of a drug. The most usual is to view the body as consisting of compartments between which drugs move and from which elimination occurs. The transfer of drugs between these compartments is represented by rate constants. Rate constants may be expressed by reactions of zero-order or first-order. Normally, all drugs are described to behaviour accordingly to a first-order reaction type. However, a continuous increase of the drug administered will change a first-order process to a zero-order process, such as occurs in an overdose situation (Dhillon and Gill, 2006).

Pharmacokinetic models are hypothetical structures that are used to describe the fate of a drug in a biological system following its administration. In general, there are three kinds of pharmacokinetic models: one-compartimental, two-compartimental and multicompartimental model. In one-compartimental models, following drug administration, the body is assumed as a kinetically homogeneous unit. This assumes that the drug achieves instantaneous distribution throughout the body and that the drug equilibrates instantaneously between tissues. The two-compartment model resolves the body into a central compartment and a peripheral compartment. Although these compartments have no physiological or anatomical meaning, it is assumed that the central compartment comprises tissues that are highly perfused such as heart, lungs, kidneys, liver and brain. The peripheral compartment comprises less well-perfused tissues such as muscle, fat and skin. A two-compartment model assumes that, following drug administration into the central compartment, the drug distributes between that compartment and the peripheral compartment. However, the drug does not achieve instantaneous distribution, i.e. equilibration, between the two compartments. In a multicompartimental model the drug distributes into more than one compartment and the concentration–time profile shows more than one exponential. Each

exponential on the concentration–time profile describes a compartment (Dhillon and Gill, 2006).

After the pharmacokinetic model is set, several useful parameters may be calculated: volume of distribution (V_d), maximal concentration (C_{max}), time on which the concentrarion is maximal (t_{max}), half-life time ($t_{1/2}$), clearance (CL), observed area under curve ($AUC_{(0\rightarrow t)}$), area under curve from administration extrapolated to infinite time ($AUC_{(0\rightarrow \infty)}$) and mean residence time (MRT) (Dhillon and Gill, 2006).

The volume of distribution (V_d) of a medication is an abstract parameter and is defined as the amount of drug in an organism divided by its plasma concentration. After distribution of the drug within the organism, the volume of distribution is computed as follows: V_d = m (total amount of drug [mg]) / C (drug plasma concentration [mg/L]). This formula can be easily understood if one considers that concentration (C) represents the quotient of the mass (m) and the volume (V). Only for a few medications, however, does the distribution volume correspond to a real physiological space, such as: the plasma volume (0.05 L/kg), the extracellular volume (0.2 L/kg) and the body water (0.66 L/kg). This is due on the one hand to the accumulation of many medications in various tissues, such as the adipose tissue or the bones (producing an increase in the numerator), and on the other hand to the avid binding with plasma proteins that prolongs the persistence of the agent within the vascular system (producing an increase in the denominator). Both are properties resulting from the solubility and the plasma-protein binding of a medication and which are constant only for one particular patient. Interindividual differences depending on the amount of body fat or body water must also be taken into consideration (Brüggmann, 2008). The total clearance (CL) of a drug consists of the renal clearance and the extra-renal clearance. For most medications the latter can be equated with hepatic clearance. As a rule one can assume that renal insufficiency only reduces the renal clearance and that liver disease only impairs the extra-renal clearance. The renal portion of the total clearance results from the so-called renal dose fraction, i.e. from that portion of an administered dose that is eliminated unchanged via the kidneys

(Brüggmann, 2008). The $AUC_{(0\rightarrow\infty)}$ represents the total drug exposure over time. Assuming linear pharmacodynamics with elimination rate constant *k*, one can show that AUC is proportional to the total amount of drug absorbed by the body (i.e. the total amount of drug that reaches the blood circulation). This is useful when trying to determine whether two formulations of the same dose (for example a capsule and a tablet) release the same dose of drug to the body. AUC becomes useful for knowing the average concentration over a time interval, AUC/t. Also, AUC is referenced when talking about elimination. The amount eliminated by the body (mass) = CL (volume / time) x AUC (mass x time / volume) (Lappin *et al.*, 2006). MRT refers to the time that a drug spends in the body. This is dependent on an individual body size, the rate at which the drug will move through and react within the person's body, and the amount of the drug administered. The MRT of a drug deviates from the previous equations as it is based on a statistical derivation. This still runs off a steady-state volume assumption but then uses the area under a distribution curve to find the average drug dose clearance time. The distribution is determined by numerical data derived from either urinary or plasma data collected. Each drug will have a different residence time based on its chemical composition and route of administration. Some of these drug molecules will remain in the system for a very short time while others may remain for a lifetime. Since individual molecules are hard to trace, groups of molecules are tracked and their distribution is plotted to find a mean residence time (Rowland and Tozer, 1995).

In the last fifteen years, substantial technological advances have been done in particle chemistry performance, improving detector design and in optimizing the system and data processors for chromatographic techniques. HPLC and GC analytical methods to determine polyphenols in different matrices have been reported. GC-MS, that is a commonly used technique and a method of choice in volatile polyphenols' analysis by using derivatization of compounds, reveals limitations, namely the correct analysis of all possible samples' compounds, including unconjugated fractions, due to its low limit of detection (LOD). Recovery yield is low and just a limited group of polyphenols can be analyzed. Many HPLC methods have been developed for the determination of polyphenols

in biological fluids, either alone or conjugated. Some of these methods were time-consuming, expensive, or there was no internal standard, being not directly applicable. HPLC-UV was optimized for the separation and identification of major conjugate metabolites, with robustness to small changes in mobile phase, and being selective, precise, accurate robust and was, then, suitable for the quantitative determination of total compounds (free and conjugated) in urine samples. Moreover, UV detection did not allow the identification of some compounds and their conjugates due to fixed wavelength limitation. Hyphenating PDA and/or MS detectors to HPLC allowed the attainment of substantially good precision and accuracy values (usually higher than 90.0%). Its developing enabled the first identification of the entire profile of some polyphenols, without reducing the resolution of glucuronides, with an accurate index of exposure and detail of the polyphenols and its metabolites and conjugates (Santos *et al.*, 2014). Beyond that, it was also possible to reduce time of analysis and ameliorate LOQ and LOD, the wide calibration range in comparison to other methods (e.g. GC) and to avoid need of enzymatic hydrolysis, simplifying the quantitative and qualitative analysis. It is considered to be an appropriate tool for further identification and quantification of polyphenols and its metabolites, in human pharmacokinetic studies. HPLC-ESI-MS method has allowed the identification and quantification of polyphenols in biological samples, conferring lowest LOD. Taken together, HPLC-MS, NMR and HPLC-PDA analysis allowed to evaluate several polyphenolic compounds in plasma and urine and broadened the spectrum of identification and quantification of new metabolites. Finally, UPLC has revealed improved sensitivity, being much faster (within 5 minutes), requiring a much lower volume of biological sample, as well as a simple sample preparation procedure, good recoveries and minor matrix effect. Many studies started experiencing separation obstacles with it. This method is valuable for human clinical studies because large volume of blood sample is usually available in humans, which may be used to concentrate the analytes before analysis.

Another important procedure in pharmacokinetic studies and its further analysis is sample preparation. When LODs and LOQs at ng/mL levels are

improved, occurs benefit in the resolution of conjugate metabolites. SPE approach is always used to gain cleaner samples and allows the LOD to be lowered 10 times, but it is time-consuming and is not economic when used to measure a large number of plasma samples in pharmacokinetic studies. So, a brand new SPE based methodology at a micro scale was developed over the classic solid-phase extraction technique. This method has shown a high specificity, a rapid isolation of analytes from complex matrices and much shorter analysis time.

Trying to catalogue analytical methods for polyphenols with the present data can be inglorious. A strong suggestion lies on searching the best method for each polyphenols group, accordingly to their physical and chemical properties, and thus, to enable a close outlook of the most suitable method.

4.1.1. Pharmacokinetic studies in rats

The rat is still the most popular animal model used by researchers all over the world, as a plausible alternative to human studies. Moreover, the metabolic/pharmacokinetic approach in clinical studies must always be preceded by robust and reliable assays in rats. In recent years, a pronounced development has been achieved in the validation of several pharmacokinetic methods in this animal model (**Table I.7**).

Carando *et al.* (1998) described the measurement of catechin in human plasma. Extraction with acetonitrile was followed by HPLC on a C_{18} column. Hollman and colleagues (1998) developed a method to analyze quercetin in human plasma and urine employing acid hydrolysis to liberate the aglycone, followed by HPLC on a C_{18} column and post-column derivatization with $AlNO_3$ to generate a fluorescent complex. Variants of this method substituting acid hydrolysis for enzymatic (glucuronidase/sulfatase) were later reported (Morrice *et al.*, 2000; Ader *et al.*, 2000). Another recent work regarding resveratrol, and also emodin, was carried out by Lin and co-workers. In this study, plasma and tissue samples were assayed by HPLC-UV before and after hydrolysis with *β*-glucuronidase and sulfatase (Lin *et al.*, 2012).

HPLC appears to be the most widely used method for quantifying plant constituents and metabolites. The solid-phase extraction (SPE) approach is always used to gain cleaner samples. This allows the LOD to be lowered 10 times, but it is time-consuming and is not economical when used to measure a large number of plasma samples in pharmacokinetic studies. The HPLC-UV method is a quick, precise and reliable method for the analysis of resveratrol in pharmacokinetic studies (He *et al.*, 2006).

Serra *et al.* presented a UPLC-MS validated method that can be used for epicatechin monitoring *in vivo*. One of the greatest advantages of the UPLC method developed is the possibility of quantifying the compound(s) studied at low concentrations within 5 minutes. This analysis time is between 2 and 5-times shorter than the methods reported in the literature for the analysis of the same kind of compounds by HPLC. Additionally, the UPLC system allowed the volume of the sample injected to be between 4 and 40 times lower than the other methods reported in the literature (Serra *et al.*, 2009).

An UPLC-MS method was properly validated by Wang and his team for the study of taxifolin kinetic behavior in rats. This method was linear over the concentration range of 6-6750 ng/mL. Intra- and inter-day precisions were all within 8% and accuracy ranged from 92.9% to 105.1%. The LOQ was 6 ng/mL. The present method was successfully applied to the estimation of the pharmacokinetic parameters of taxifolin following intravenous and oral administration to rats (Wang *et al.*, 2009).

Marti and co-workers (Martí *et al.*, 2010) developed a rapid, simple and sensitive method to simultaneously extract and analyze procyanidins and anthocyanins, and their metabolites in plasma samples by microelution SPE (μSPE) plates and UPLC-ESI-MS, respectively. This extraction methodology allows to prepare 96 samples at one time and to increase sample throughput. On the other hand, Urpi-Sardà *et al.* (2009) used 96-well plates with millielution SPE (mSPE) to analyze procyanidins in rat plasma after cocoa intake. The main difference between these two designs is the capacity of the sorbent in SPE, which in the case of mSPE is from 5 to 60 mg and in μSPE plate is only 2 mg.

In the analysis of biological samples, such as plasma or serum, the quantity of sample available is a critical parameter in most cases. The μSPE plate can be a good approach because it allows the rapid isolation of the analytes from complex matrices because lower sample volume is loaded into the plate and lower elution solvent is necessary to elute the retained analytes. Moreover, in this design, the solvent evaporation and reconstitution steps are eliminated, and as a consequence the time for sample preparation is significantly reduced.

In what concers HPLC-MS methodologies, Prasain and co-workers (2004) reported a remarkable LOD and a good calibration range, while He and colleagues (2006) using SPE technique improved the LOD, being 10 times better than previous studies. This method proved to be very precise. The method developed by Ma and collaborators (Ma *et al.*, 2006), presented a remarkable calibration range and linearity, and it is very precise. Jan and colleagues (2009) reported a method with very good linearity, precision and accuracy, while the method from Wang and his team (2009) showed a very wide calibration range.

Serra *et al.* (2009) introduced the UPLC methodology to improve the sensitivity, in addition for being much more rapid and to require a volume of biological sample 40 times lower. The type of columns used in this methodology is a substantial step forward to the pharmacokinetic studies of all analytes. Kapetanovic and co-workers (2011) presented recently a validated method with very good precision and accuracy, in a very wide range. Finally, the method developed by Lin and colleagues (2012) exhibited very good precision and accuracy values, but not so good extraction recoveries.

Few studies have been performed on the absorption, tissue distribution, metabolism and excretion of flavone *C*-glycosides (Sheng *et al.*, 2014; Xue *et al.*, 2014). Understanding the biodynamics of flavone *C*-glycosides after oral administration is fundamental to understand the existing structure-activity relationship and its potential use as preventive of some diseases. In addition to structural and physical-chemical attributes of the original compound, the absorption, pharmacokinetics and biotransformation to certain metabolites are crucial determinants of biological effects in organisms. However, the current

understanding of absorption and metabolism is limited to a small number of flavonoids and few studies refer to the metabolism of flavone *C*-glycosides (Zhang *et al.*, 2007). Recently, the pharmacokinetic profile of vitexin glycosides has been studied (Ma *et al.*, 2010; Zhang *et al.*, 2010). Intact forms of vitexin *O*-glucoside and *O*-rhamnoside were detected in plasma, tissues, urine and bile after a single oral dose of a crude extract (Ma *et al.*, 2010). This work also showed that deconjugation of *C*-glycosyl flavone is not a prerequisite for its absorption in rats. Puerarin, a *C*-glycosyl flavone, could be rapidly absorbed from the intestine without metabolism after oral administration (Prasain *et al.*, 2004). High recovery of administered vitexin 4''-*O*-glucoside and 2''-*O*-rhamnoside in faeces indicated that the efficient absorption of these two molecules from the gastro-intestinal tract of rats was low. It was reported that vitexin 2''-*O*-rhamnoside had limited gastrointestinal absorption with an oral bioavailability of about 3.57% (Liang *et al.*, 2007). Another work investigated the pharmacokinetic behavior of six compounds: three secoiridoid glycosides and three flavonoid *C*-glycosides (Sheng *et al.*, 2014). This study indicated that the bioavailability of the secoiridoid glycosides was much higher than that of flavonoid glycosides. The time to reach C_{max} (T_{max}) ranged from 10 to 40 minutes, and the elimination half-life ($T_{1/2}$) ranged from 58.4 to 263.0 minutes. These results demonstrate that both the absorption and elimination processes of the six analytes were fast. This work also showed that the six analytes are mainly excreted by urine. The amounts of the six analytes excreted by bile, urine and faeces were very limited (<5.0%), suggesting that the six analytes were mainly excreted as metabolites.

Two new metabolites (dihydrodaidzein and equol) of a *C*-glycoside flavone, the puerarin, as well as its mono- and dihydroxylated derivatives were investigated, using a HPLC-ESI-MS/MS validated method by Prasain *et al.* (2004). These metabolites were detected in both urine and faeces of rats after oral administration. However, the persistence of puerarin in the blood and urine as the principal metabolic form for the period of 4-72 hours after oral administration suggested that puerarin is rapidly absorbed from the intestine without being metabolized.

Table I.7. Validated bioanalytical methods for the pharmacokinetic study of polyphenols in rats (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Resveratrol	Resveratrol	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 1-2500μg/L Precision >92.9% LOD – 0.01μg/L LOQ – 0.1μg/L Recovery >79.6%	Soleas, Yan & Goldberg, 2001
	Resveratrol Resveratrol glucuronide Resveratrol sulfate	Intragastric Oral	Plasma Tissues	HPLC-UV	Plasma: Regression type – linear fit Calibration range – 0.16-20μg/mL Precision >90.1% Accuracy >90.4% LOD – 0.08μg/mL LOQ – 0.16μg/mL Recovery – 65-98% Tissues: Regression type – linear fit Calibration range – 0.08-2.5μg/mL Precision >86% Accuracy >90.4% LOD – 0.04μg/mL LOQ – 0.08μg/mL Recovery – 88-112%	Lin, Chu, Tsai, Wu & Hou, 2012
	Resveratrol	Oral IV	Plasma	HPLC-MS	Regression type – linear Calibration range – 5-1000ng/mL Precision >90% Accuracy – 95-105%	Kapetanovic, Muzzio, Huang, Thompson & McCormick, 2011
	Resveratrol	Oral	Plasma	HPLC-UV	Regression type – linear Calibration range – 0.02-40μg/mL Precision >88% LOQ – 0.02μg/mL Recovery – 84.4-98.7%	He, Chen, Wang, Wang & Davey, 2006

Table I.7. Validated bioanalytical methods for the pharmacokinetic study of polyphenols in rats (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Pterostilbene	Pterostilbene	Oral IV	Plasma	HPLC-MS	Regression type – linear Calibration range – 5-1000ng/mL Precision >90% Accuracy – 101-103%	Kapetanovic, Muzzio, Huang, Thompson & McCormick, 2011
Sesaminol triglucoside	Sesaminol triglucoside Sesaminol Sesaminol glucuronide Sesaminol sulfate	Intragastric	Faeces Plasma Tissues Urine	HPLC-UV	Regression type – linear Calibration range – 0.02-25μg/mL Precision – 97.1-99.9% Accuracy – 96.1-104.8% LOQ – 0.4μg/mL	Jan, Hwang & Ho, 2009
Naringenin	Naringenin	Oral	Plasma	HPLC-MS	Regression type – linear Calibration range – 5-2500ng/mL Precision >92.2% Accuracy – 97.60-100.34% LOQ – 5ng/mL Recovery >81%	Ma, Li, Chen, Fang, Li & Su, 2006
Quercetin	Quercetin	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 1-2500μg/L Precision >87% LOQ – 0.1μg/L Recovery >81%	Soleas, Yan & Goldberg, 2001
Taxifolin	Taxifolin	Oral	Plasma	UPLC-MS	Regression type – linear Calibration range – 6-6750ng/mL Precision >92% Accuracy – 92.9-105.1% Recovery >75%	Wang, Xia, Xing, Deng, Shen & Zeng, 2009
Puerarin	Puerarin (Di)Hydroxylated Daidzein Dihydrodaidzein Equol	Intragastric	Faeces Plasma Tissues Urine	HPLC-MS	Regression type – linear Calibration range – 0.05-50μM Precision >85%	Prasain, Jones, Brissie, Moore, Wyss & Barnes, 2004

Table I.7. Validated bioanalytical methods for the pharmacokinetic study of polyphenols in rats.

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Epicatechin	Epicatechin	Intragastric	Plasma	UPLC-MS	Regression type – linear fit Calibration range – 0.02-3μM Precision – 91.4-96.1% Accuracy – 99.8% LOD – 0.004μM LOQ – 0.015μM Recovery – 96%	Serra *et al.*, 2009
Procyanidins	Catechin Epicatechin Dimer B_2 Trimer	Intragastric	Plasma	UPLC-MS	Regression type – linear fit Calibration range – 0.04-11.5μM Precision – 95.2-99.4% Accuracy – 95-105% LOD – 0.007-0.70μM LOQ – 0.010-1.38μM Recovery – 81-100%	Marti *et al.*, 2010
Pelargonidin	Pelargonidin Glucuronide 4-Hydroxy-benzoic acid	Oral	Faeces Plasma Tissues Urine	HPLC-MS	Regression type – linear Calibration range – 0.5-20μg/mL Recovery – 90%	El Mohsen *et al.*, 2006

4.1.2. Pharmacokinetic studies in humans

Human model gives the best approach for drug pharmacokinetic profile studies. In the last decade, the number of bioanalytical and pharmacokinetic studies with human plasma, blood and urine have been enhanced (**Table I.8**). This assumes the major importance, due to the possibility of the best dose prediction and polyphenols' pharmacokinetic behavior following *in vivo* administration, in relation to other animal species used, and also in *in vitro* studies, such as in cell cultures. Compared with the effects of polyphenols *in vitro*, the results *in vivo*, although significant, are more limited (Serra *et al.*, 2013). The reasons for this are: 1) lack of validated *in vivo* biomarkers; 2) lack of long-term studies; and 3) lack of understanding or consideration of bioavailability in the *in vitro* studies, which are subsequently used for the design of *in vivo* experiments (Manach *et al.*, 2005).

The bioavailability and, consequently, bioactivity of polyphenols are strongly determined by the gut metabotype of each individual and personalized screenings, and applications are therefore highly recommended (Bolca *et al.*, 2013; Schröder *et al.*, 2013). The composition and metabolic activities of the human intestinal flora differs from that of experimental animals (Hirayama and Itoh, 2005). Animals with human flora-associated (HFA) have been used for studying the metabolism of the human intestinal flora. Still, results with HFA animals suggest that the role of the human intestinal flora is somewhat different from the role of the animal flora in conventional experimental animals (Zeng *et al.*, 2013).

Analyses of blood, and even less so of plasma, may not be considered as representative for the whole organism, as soluble metabolites are bound to be enriched in the blood aqueous phase, while less hydrophilic ones or the parent compounds may associate more readily with other tissues, depending on characteristics such as lipid content and accessibility (Biasutto *et al.*, 2010). In many cases, the assessment of levels in tissues has relied on the enzymatic conversion of conjugates to the aglycone before quantification.

The concentrations of catechin, quercetin and resveratrol in samples such as blood serum, plasma, urine and whole blood have been measured following their oral and intragastric administration to humans. These compounds and their

conjugates were analyzed by GC analysis followed by MS detection employing two target ions and one qualifier ion for each compound. Excellent resolution and linearity was obtained for all three polyphenols in all biological fluids sampled (Soleas *et al.*, 2001).

In a study about curcumin, Ireson and colleagues developed an HPLC-MS method to characterize its metabolites *ex vivo*. The major metabolites in suspensions of human hepatocytes were identified as hexahydrocurcumin and hexahydrocurcuminol (Ireson *et al.*, 2001).

In another study with volunteers, they were fed with a diet containing catechin, catechin, 3'-*O*-methylcatechin, and metabolites of microbial origin were identified in urine. Catechin and its 3'-*O*-methylated derivative formed in the small intestine and liver were the most abundant compounds excreted and accounted for 25.7% of the catechin ingested. This finding agrees with recoveries of 20.0–40.0% reported in previous studies and confirms the good absorption level of catechin by the small intestine. On the other hand, aromatic acids formed from catechin accounted for 10.6% of the catechin ingested (Gonthier *et al.*, 2003).

A solid-phase extraction reversed-phase HPLC-UV assay for the simultaneous determination of hesperetin and naringenin in human urine was developed and validated. Urine samples were incubated with glucuronidase/sulphatase. Stability studies showed urine quality control samples to be stable for both hesperetin and naringenin through three freeze–thaw cycles and at room temperature for 24 hours (error ≤ 3.6%). This method was selective, precise, accurate and robust, and seemed to be suitable for the quantitative determination of total (free and conjugated) hesperitin in urine samples obtained in pharmacokinetic studies in humans, after oral administration of the dietary flavanone glycosides, hesperidin and naringin, either as pure substances or in citrus fruits that contain them. The determination of urinary flavanone aglycone hesperitin may be an useful biomarker for flavonoids intake (Kanaze *et al.*, 2004).

A sensitive, precise and selective analytical method has been developed for the identification and quantification of resveratrol metabolites in human low-density lipoprotein (LDL) after moderate consumption of red wine, using HPLC-

ESI-MS/MS. Results to date indicate that resveratrol metabolites were incorporated into LDL after a moderate intake of red wine. The metabolites identified in LDL were *trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide and *cis*-resveratrol-3-*O*-glucoside, as well as free *trans*-resveratrol (Urpí-Sardà *et al.*, 2005).

A rapid, sensitive and accurate protein precipitation procedure and sensitive HPLC separation coupled to UV detection was described allowing quantification of resveratrol and separation and identification of at least six major conjugate metabolites in human plasma and urine. The fast and non-intensive sample preparation method by protein precipitation simplifies large sample processing for clinical trials, having better or similar LOQ for resveratrol (5 ng/mL), and resolving the major conjugate metabolites (Boocock *et al.*, 2007).

Urpí-Sarda *et al.* (2007) described an HPLC-MS method to characterize the metabolic profile of resveratrol in human urine and LDL after sample clean-up with SPE, to study resveratrol and its metabolites bioavailability. This method enabled the determination of resveratrol metabolic profile in 10 minutes for different types of matrices. To improve the resolution of the sulfates, acetone was incorporated into the mobile phase. Acetone allows better resolution of sulfates by improving the peak shape and reducing the relative retention time. The incorporation of a shorter chromatographic column also reduced the chromatographic time to 10 min. The use of a 96-well SPE plate helped to avoid laborious sample preparation, requiring ~3 hours of preparation per plate. The use of HPLC-MS/MS avoids the need to perform enzymatic hydrolysis, thus simplifying the qualitative and quantitative profiling of the resveratrol metabolites. Another highlight of the present method is the ability to differentiate between the *trans* and *cis* isomers of resveratrol-4-*O*-glucuronide, resveratrol-3-*O*-glucuronide, resveratrol-4-sulfate and resveratrol-3-sulfate. This method is the first to identify the entire profile of resveratrol sulfates in human LDL and urine, and furthermore, without reducing the resolution of glucuronides (Urpi-Sarda *et al.*, 2007).

The soy isoflavone, daidzein, was studied in a human trial after oral administration of both aglycone and glucoside forms. Isoflavonoid trimethylsilyl

ether derivatives were separated and quantified by GC-MS analysis. The limits of detection were between 8 and 70 fmol. The recoveries ranged between 93% and 105% obtained after spiking plasma samples with reference compounds and subtracting the basal values from the blank plasma. The intra- and inter-assay coefficients were always <5% for all quantified isoflavonoids. This area ratio was then interpolated against calibration curves in the linear range between 0 and 25 pmol constructed for known amounts of the individual isoflavonoids. It was not possible to determine the unconjugated fractions of the oxidative metabolites in plasma because the free plasma concentrations were below the limit of detection (Rüfer *et al.*, 2008).

Before questions about polyphenols' bioavailability can be addressed, ultrasensitive methods are necessary to study their pharmacokinetics, which is not yet linear. In fact, these studies involve several parameters, like the dose and nature of the phenolic compound, and procedures capable of influencing final results and conclusions. Additionally, the lack of analytical parameters and characteristics in published papers with analytical validation of the procedure is frequent, hindering viable comparisons between studies. Bioavailability and pharmacokinetics studies of polyphenols have been assessed mostly through human and animal models, particularly rodents and primates, and more recently, the nematode *Caenorhabditis elegans* has been successfully used in toxicological and in tissue distribution studies. It is recommended to increase the length of human intervention studies, to more closely reflect the long-term consumption of polyphenols. Together with validated *in vivo* biomarkers, these issues will contribute to achieve a better *in vitro*/*in vivo* bioavailability correlation.

Table I.8. Validated bioanalytical methods for the study of polyphenols in humans (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Resveratrol	Resveratrol	Oral	Plasma Urine	HPLC-UV	Plasma Regression type – linear fit Calibration range – 5-500 ng/mL Accuracy – 94.6-104.4% Precision – 90.3-96.2% LOD – 2 ng/mL LOQ – 5 ng/mL Recovery – 58±3% Urine Regression type – linear fit Calibration range – 5-1000 ng/mL Accuracy – 98.8-104.1% Precision – 89.6-96.8% LOQ – 5 ng/mL Recovery – 58±3%	Boocock *et al.*, 2007
	Resveratrol	Oral	Plasma	HPLC-MS	Regression type – linear Calibration range – 0.5-100 ng/mL Precision >89.2% Accuracy – 88.5-91.8% LOQ – 0.5 ng/mL Recovery – 76.8%	Almeida *et al.*, 2009
	Resveratrol Glucuronides Glucoside	Oral	LDL	HPLC-MS	Regression type – linear fit Calibration range – 0.44-438.59 pmol/mL Precision >94% Accuracy – 98.7-99.7% LOD – 0.15 pmol/mL LOQ – 0.44 pmol/mL Recovery – 87±4%	Urpí-Sarda *et al.*, 2005

Table I.8. Validated bioanalytical methods for the study of polyphenols in humans (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Resveratrol	Resveratrol Glucuronides	Oral	Plasma Urine	HPLC-MS ^{13}C NMR	Regression type – linear Precision > 95% LOQ – 0.02 μg/mL (plasma); 0.05 μg/mL (urine) Recovery >97%	Burkon & Somoza, 2008
	Resveratrol	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 1-2500 μg/L Precision >92.9% LOD – 0.01 μg/L LOQ – 0.1 μg/L Recovery >79.6%	Soleas, Yan & Goldberg, 2001
	Resveratrol Glucosides Glucuronides Sulfates Taxifolin Hexestrol	Oral	LDL Urine	HPLC-MS	Regression type – linear Calibration range – 4.4-3289.5 nM Precision >89.2% LOD – 0.2 and 1.2 nM (LDL matrix); 4.0 and 8.4 nM (urine) LOQ – 4.4 and 12.8 nM (urine); 0.4 and 1.9 nM Recovery – 89-92%	Urpí-Sarda *et al.*, 2007
Curcumin	Curcumin Curcumin glucuronide Curcurmin sulfate Hexahydrocurcumin Hexahydrocurcuminol	Intragastric	Plasma	HPLC-MS	Regression type – linear Calibration range – 20 nM-40 μM Precision – 90.2-94.9% LOD – 5-10 nM LOQ – 20 nM	Ireson *et al.*, 2001

Table I.8. Validated bioanalytical methods for the study of polyphenols in humans (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Dimethoxycinnamic acid	Dimethoxycinnamic acid Dimethoxycinnamoyl-quinic acids Caffeoyl/feruloyl-dimethoxycinnamoyl-quinic acids di-esters	Oral	Plasma	HPLC-MS	Regression type – linear fit Calibration range – 150-4800 nM Precision – 92-96% Accuracy >90% LOD – 50 nM LOQ – 150 nM Recovery – 94-105 %	Farrell *et al.*, 2012
Chlorogenic acid	Chlorogenic acid 3,4-Didhydroxycinnamic acid Cinnamic acid Phenylpropionic acid Benzoic acid Quinic acid Cyclohexane carboxylic acid 3-Methoxy-4-hydroxybenzoate Hippuric acid	Oral	Plasma Urine	GC-MS	Precision – 88-96% LOD – 4 mg/L Recovery >70%	Olthof, Hollman, Buijsman, van Amelsvoort & Katan, 2003
Hesperidin	Hesperitin	Oral	Urine	HPLC-UV	Regression type – linear Calibration range – 50-1200 ng/mL Precision >91.8% Accuracy – 95.5-103.4% LOQ – 50 ng/mL Recovery >73.1%	Kanaze, Kokkalou, Georgarakis & Niopas, 2004

Table I.8. Validated bioanalytical methods for the study of polyphenols in humans (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Naringin	Naringenin	Oral	Urine	HPLC-UV	Regression type – linear Calibration range – 50-1200 ng/mL Precision >92.2% Accuracy – 94.9-101.5% LOQ – 50 ng/mL	Kanaze, Kokkalou, Georgarakis & Niopas, 2004
Quercetin	Quercetin	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 1-2500 μg/L Precision >87% LOD – 0.01 μg/L LOQ – 0.1 μg/L Recovery >81.0%	Soleas, Yan & Goldberg, 2001
Quercetin 3-*O*-rutinoside	Quercetin 3-*O*-rutinoside Quercetin 3,4-Hydroxyphenylacetic acid 3-Hydroxyphenylacetic acid Isorhamnetin 3-Methoxy-4-hydroxyphenylacetic acid	Oral	Plasma Urine	GC-MS	Precision – 88-96% LOD – 0.4 mg/L Recovery >70%	Olthof, Hollman, Buijsman, van Amelsvoort & Katan, 2003
Daidzein	Daidzein Daidzin Dihydrodaidzein 8-Hydroxydaidzein 6-Hydroxydaidzein 3'-Hydroxydaidzein Daidzein 7-*O*-glucuronide Daidzein 4'-*O*-sulfate	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 0-25 pmol Precision >95% LOD – 8-70 fmol Recovery >93%	Rufer, Bub, Moseneder, Winterhalter, Sturtz & Kulling, 2008

Table I.8. Validated bioanalytical methods for the study of polyphenols in humans (cont.).

Polyphenol	Analyte(s)	Administration Route	Biological Sample	Method	Validation Parameters	Reference
Daidzin	Daidzein Daidzin Dihydrodaidzein 8-Hydroxydaidzein 6-Hydroxydaidzein 3'-Hydroxydaidzein Daidzein 7-*O*-glucuronide Daidzein 4'-*O*-sulfate Equol	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 0-25 pmol Precision >95% LOD – 8-70 fmol Recovery >93%	Rufer, Bub, Moseneder, Winterhalter, Sturtz & Kulling, 2008
Catechin	Catechin	Oral	Plasma Urine	GC-MS	Regression type – linear Calibration range – 1-2500 μg/L Precision >92.9% LOD – 0.01 μg/L LOQ – 0.1 μg/L Recovery >86.8%	Soleas, Yan & Goldberg, 2001
Catechin	Catechin 3,4-Dihydroxyphenyl-valeric acid 3-Hydroxyphenyl-valeric acid 3,4-Dihydroxyphenyl-propionic acid 3-Hydroxyphenyl-propionic acid Protocatechuic acid Vanillic acid 4-Hydroxybenzoic acid 3-Hydroxybenzoic acid *m*-coumaric acid Ferulic acid	Oral	Urine	HPLC-MS	Regression type – linear Calibration range – 0.125-6 μM Precision >91.2% Accuracy – 97.7% LOD – 0.01 μg/L LOQ – 0.125 μM Recovery – 55-86%	Gonthier, Rios, Verny, Remesy & Scalbert, 2003

LOD: limit of detection; LOQ: limit of quantification.

C. Aims

Infusion of *C. citratus* leaves 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 mechanisms of action and its bioactive compounds. On the other hand, the development of new anti-inflammatory drugs is of utmost importance, since inflammation has been associated with several pathologies, including Alzheimer, diabetes, cardiovascular diseases, atherosclerosis and cancer, but current anti-inflammatory drugs are not totally safe and effective. During the last decade, developments have been achieved on the phytochemical characterization (Figueirinha *et al.*, 2010, 2008) and the disclosure of the anti-inflammatory mechanisms of lemongrass infusion *in vitro* (Francisco *et al.*, 2014, 2013, 2011). In contrary, little attention has been paid to the *in vivo* approach.

Therefore, the aims of the present study are to:

- Develop and validate an analytic method to quantify the phenolic compounds in *C. citratus* extracts;
- Investigate the influence of harvest time and plant quality on *C. citratus* infusion phenolic compounds profile;
- Further elucidate *C. citratus* phenolic compounds chemistry, particularly of the tannins;
- Evaluate the potential of *C. citratus* infusion as source of new anti-ulcer and/or anti-inflammatory drugs, using *in vivo* bioguided assays;
- Assess the contribution of phenolic compounds - flavonoids and tannins - to the overall *C. citratus* activity;
- Incorporate *C. citratus* infusion and polyphenol-enriched fractions in a pharmaceutical formulation with anti-inflammatory activity;
- Study the pharmacokinetics in an *in vivo* model of phenolic compounds from *C. citratus* infusion.

RESULTS and DISCUSSION

CHAPTER II

A. Validation of a RP-HPLC method for quantitation of phenolic compounds in three different extracts from *Cymbopogon citratus*

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Abstract

Cymbopogon citratus (lemongrass) leaves and/or infusion are commonly consumed as a beverage or as an herb in several cuisines worldwide. It is also used in traditional medicine for the treatment of several pathologic conditions, such as peptic ulcers, inflammatory conditions and cancer. Recent studies revealed the crucial role of the phenolic compounds of the infusion bioactivity as anti-inflammatory and antineoplasic, particularly attributed to flavonoids. A high-performance liquid chromatography – diode-array detector (HPLC-DAD) method was developed, for the first time, for the simultaneous quantification of phenolic compounds in *Cymbopogon citratus* polar extracts, which seem to be the main contributors to the antioxidant effects of this herb. The separation was performed on a Spherisorb S5 ODS-2 column (250 x 4.6 mm i.d.; particle size, 5 μm), using a methanol: aqueous formic acid (5%, v/v) gradient as the mobile phase, with a flow of 1 mL min^{-1}. The detection was achieved with a DAD detector and the chromatographic profiles were registered at 280 nm. The developed method was validated according to the Food and Drug Administration (FDA) recommendations. Good linearity was observed within a wide concentration range. The developed method is simple, precise at the intra-day and inter-day levels and accurate with very good recovery percentages. Phenolic acids and flavonoids, including luteolin 6-*C*-glucosyl-8-*C*-arabinoside, luteolin 6-*C*-glucoside, luteolin 7-*O*-glucoside, luteolin 7-*O*-neohesperidoside, luteolin 6-*C*-arabinosyl-2''-*O*-rhamnoside and luteolin 2''-*O*-rhamnosyl-*C*-(6-deoxy-ribo-hexos-3-ulosyl) were successfully quantified in leaves infusion of *C. citratus* using the developed HPLC method. This data may be of great interest for the food supplement and pharmaceutical industries.

Keywords: Lemongrass, polyphenols, flavonoids, HPLC.

1. Introduction

Cymbopogon citratus (DC.) Stapf, Poaceae, commonly known as lemongrass, is a spontaneous perennial graminoid, largely distributed in tropical and subtropical countries. The lemon-like flavour of the plant is responsible for its use in tropical countries cuisines being its leaves a common ingredient in Asian cuisine in teas, soups and curries, being also suitable for fish, seafood and poultry (Adeneye and Agbaje, 2007). In African and Latin American countries, this herb is highly consumed as an aromatic and pleasant-tasting herbal drink (Francisco *et al.*, 2014). In traditional medicine, aqueous extracts of lemongrass leaves are also used for the treatment of several inflammation-based pathologies (Shah *et al.*, 2011), cardiovascular diseases (Runnie *et al.*, 2004), cancer (Puatanachokchai *et al.*, 2002), digestive disorders (Bergonzelli *et al.*, 2003), nervous system disorders (Linck *et al.*, 2010), infections (Barbosa *et al.*, 2008), among other health problems.

Preliminary results obtained by our group (Figueirinha *et al.*, 2010) proved that an essential oil-free infusion from *C. citratus* leaves induced a reduction of nitric oxide (NO) production in dendritic cells co-incubated with an inflammatory stimulus (lipopolysaccharide - LPS), allowing cell viability under the assayed conditions. In another recent work, we have demonstrated that *C. citratus* leaves extract has a potent anti-inflammatory 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 iNOS expression in fetal skin-derived dendritic cell line (FSDC) (Figueirinha *et al.*, 2010), stressing the use of *C. citratus* extract as a potential new anti-inflammatory drug. Moreover, we have previously demonstrated that *C. citratus* and its polyphenols inhibited NO production, in dendritic cells and mouse macrophages, through modulation of p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) 1/2 and NF-κB signaling pathways (Francisco *et al.*, 2013; Francisco *et al.*, 2011). More recently, the analgesic and anti-inflammatory activities of lemongrass infusion were validated in rats (Garcia *et al.*, 2015).

Studies considering chemical composition of lemongrass have shown a wide variety of phenolic compounds such as phenolic acids, condensed tannins and flavonoids, such as luteolin and apigenin derivatives (Campos *et al.*, 2014; Figueirinha *et al.*, 2008; Tavares *et al.*, 2015). Luteolin and its glycosides have been described by numerous authors by their antioxidant (Cheel *et al.*, 2005; Orrego *et al.*, 2009; Tapia *et al.*, 2007) and anti-inflammatory properties (Chen *et al.*, 2004; Costa *et al.*, 2012; Francisco *et al.*, 2014; Xagorari *et al.*, 2002). On the other hand, although there has been a few works, in the literature, regarding the quantification of the phenolic compounds of lemongrass extracts (Figueirinha *et al.*, 2008; Roriz *et al.*, 2014), there are no reports about the validation of analytical methods for the quantification of any phenolic constituents found in *Cymbopogon citratus* polar extracts.

The aim of the present study was to develop and validate a simple and sensitive reversed phase-high performance liquid chromatography (RP-HPLC) method with UV detection for the simultaneous determination of the phenolic compounds present in three different extracts from *Cymbopogon citratus* leaves.

2. Materials and Methods

2.1 Plant material and Chemicals

Cymbopogon citratus dried leaves were purchased from ERVITAL® (Castro d'Aire, 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 (Life Sciences Department, University of Coimbra, Portugal). A lipid- and essential oil-free infusion (CCI) was prepared as previously described (Figueirinha *et al.*, 2008). CCI was prepared by adding boiling water to the plant material, the mixture being kept hot and left to stand for 15 min. Two hydro-alcoholic extracts were also prepared. A maceration process was applied to 5g of dried leaves, by adding 150 mL of 50% ethanol and absolute ethanol (CCM50 and CCM100, respectively), and left to stand for 10 days, in the dark at room temperature. All extracts were

then filtered under vacuum, concentrated and freeze-dried. The freeze-dried extracts were kept at -20ºC until use. Caffeic acid (>99%) and *p*-coumaric acid (>99%) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Isoorientin (>99%) and isovitexin (>99%) were purchased from Extrasynthese (Genay, France). All other reagents and solvents were of HPLC grade and were purchased from Merck® (Darmstadt, Germany). Water for HPLC was purified with the Millipore® Simplicity System (Bedford, MA, USA).

2.2 Preparation of stock and standard solutions

Caffeic and *p*-coumaric acids stock solutions were prepared at a concentration of 10.00 μg mL^{-1}, in 50% methanol. Five standard solutions of each standard were prepared by appropriate dilution of the stock solution with mobile phase (methanol-5% (v/v) aqueous formic acid, 5:95, v/v): 5.00, 2.50, 1.00, 0.50 and 0.25 μg mL^{-1}. Isoorientin and isovitexin stock solutions were prepared at a concentration of 250.00 μg mL^{-1}, in 50% methanol. Five standard solutions of each standard were prepared by appropriate dilution of the stock solution with mobile phase (methanol-5% (v/v) aqueous formic acid, 5:95, v/v): 125.00, 62.50, 31.25, 15.63 and 7.81 μg mL^{-1}. Standard solutions were freshly prepared each day and stored at 4 ºC.

2.3 Instrumentation and chromatographic conditions

HPLC analyses were performed in a chromatograph GILSON equipped with a diode-array detector (DAD) (Gilson® Electronics SA, Villiers le Bel, France). The studies were carried out on a Spherisorb S5 ODS-2 column (250 x 4.6 mm i.d.; particle size, 5 μm; Waters® Corp., Milford, MA, USA) and a Nucleosil C18 guard cartridge (30 x 4 mm i.d.; particle size, 5 μm; Macherey-Nagel, Düren, Germany) at 25ºC. A mobile phase of 5% (v/v) aqueous formic acid (A) and methanol (B) was used with a discontinuous gradient: 5–15% B (0–10 min.), 15–30% B (10–15 min.), 30–35% B (15–25 min.), 35–50% B (25–35 min.) and 50–80% B (35–40 min.), followed by an isocratic elution for 20 min, at a flow rate of 1mL min^{-1}. An injection volume of 100 μL was used for all standards and samples.

Chromatographic profiles were acquired in the wavelength range 200–600 nm, and recorded at 280 nm. Data treatment was carried out with Unipoint® 2.10 software (Gilson®).

2.4 Method validation

The HPLC-DAD method was validated according to the US Food and Drug Administration (FDA) regulations (FDA, 2013), including also some complementary aspects taken in the guidelines from the International Conference on Harmonization (ICH) (ICH, 2005). The parameters considered for the validation included linearity, accuracy, precision, limits of detection and quantification.

2.4.1. Linearity

Calibration curves were constructed with six standard solutions, containing caffeic acid, *p*-coumaric acid, isoorientin and isovitexin. Linearity was determined through the calculation of a regression line by the method of least squares, representing the peak area as a function of the standard concentration. The *F*-test was used to evaluate the linear relationship (Araujo, 2009). The Fisher variance ratio or *F*-test ($F_{calculated}$) was calculated by the equation:

$$F_{calculated} = \frac{\sigma_{lof}^2}{\sigma_{\varepsilon}^2} = \frac{\frac{SS_{lof}}{DF_{lof}}}{\frac{SS_{\varepsilon}}{DF_{\varepsilon}}} = \frac{\frac{\sum_{i=1}^{I}(\bar{y}_i - \hat{y}_i)^2}{(I-2)}}{\frac{\sum_{i=1}^{I}\sum_{j=1}^{J_i}(y_{ij} - \bar{y}_i)^2}{(IJ-I)}}$$

where: σ_{lof}^2 is the lack-of-fit variance; σ_{ε}^2 is the purely experimental variance; SS_{lof} is the lack-of-fit sum squares; DF_{lof} is the lack-of-fit degrees of freedom; SS_{ε} is the purely experimental sum squares; DF_{ε} is the purely experimental degrees of freedom; I is the number of i concentration levels; $\bar{y}_i$ is the average response for the level i; $\hat{y}_i$ is the estimated experimental response for the level i; J is the total number of j injections per i concentration levels. The value of $F_{calculated}$ was compared against the critical value of F found in statistical tables ($F_{tabulated}$). Data collected were

analyzed using the Analysis ToolPak of Microsoft Excel® (Microsoft Corp., Redmond, WA, USA).

2.4.2. Limits of detection and quantification

The limits of quantification (LOQ) and detection (LOD) were calculated experimentally, using lowering spiking assays, with LOQ and LOD being the amount of compound that can be quantified with a signal-noise ratio of 10, and 3.3, respectively (ICH, 2005).

2.4.3. Precision and accuracy

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of matrix (FDA, 2013). Repeatability (intra-day precision) was determined by calculating the percentage of relative standard deviation (% R.S.D.) for three independent determinations at three concentrations (0.50, 2.50 and 10.00 μg mL^{-1}, for caffeic acid and *p*-coumaric acid; 7.81, 62.50 and 250.00 μg mL^{-1}, for isoorientin and isovitexin). Three independent samples were spiked with caffeic acid, *p*-coumaric acid and isoorientin and the analyte was quantified within the same day. To determine the intermediate precision the same method was applied and the samples were analyzed at the first, the third and the fifth day. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte (FDA, 2013). Intra-day accuracy was assessed by calculating the percentage of recovery according to the following equation: %accuracy = (measured concentration/nominal concentration) x 100. Intermediate precision and accuracy were assessed by assaying three standard sample levels in triplicate on three separate occasions. The %RSD should not exceed 15%, with the exception of LOQ, where it should not deviate more than 20% (FDA, 2013).

2.5 Method applicability

The developed and validated method was applied to the quantification of phenolic acids and flavonoids, namely carlinoside, isoorientin, cynaroside, luteolin 7-*O*-neohesperidoside, kurilensin A and cassiaoccidentalin B, in three different extracts of *C. citratus* leaves. It should be emphasized that it is the first time that these flavonoids are quantified by a validated method in this species. On the other hand, the simultaneous quantification and validation of these analytes, within only one run, is of remarkable importance from a time-saving and quality control perspective. For that, three independent sample solutions at 3 mg mL^{-1} of CCI, CCM50 and CCM100 were prepared from the freeze-dried extract, in 50% methanol. Calibration curves were prepared by injecting different concentrations of standard samples, recording their peak areas and plotting peak areas *vs.* concentration of the standard. For interpretation of results, the calibration curves were used to quantify the phenolic acids and flavonoids previously identified by HPLC-MS^n (Figueirinha *et al.*, 2008) in lemongrass infusion.

3. Results and Discussion

3.1 Method validation

3.1.1. Linearity

Linearity was evaluated over the concentration range of 0.50 – 10.00 μg mL^{-1} for caffeic and p-coumaric acids and over the concentration range of 7.81 – 250.00 μg mL^{-1} for isoorientin, estimating the regression equations (caffeic acid: y = 9957547x - 1980541; p-coumaric acid: y = 65289976x + 522431; isoorientin: y = 1939071x – 4248388; isovitexin: y = 19173001x + 1904129) and the determination coefficient (R^2 = 1.00) from the least squares method, for all standards (**Table II.1**). Although the coefficient of determination for the calibration curve had been higher than 0.999, only an appropriate statistical method can evaluate the acceptability of the linear model (Araujo, 2009). In this work, the *F*-test was used to evaluate the linearity. The comparison between the calculated and tabulated Fisher ratio ($F_{calculated}$ and $F_{tabulated}$, respectively) indicates that $F_{calculated}$ is lowest than the $F_{tabulated}$ (3.26 (tabulated at the 95% with 4 and 12

degrees of freedom)), which is considered an evidence of an acceptable fit of the data to the regression line and indicating a good linearity over the concentration range proposed.

3.1.2. Limits of detection and quantification

The experimentally determined limit of detection (LOD) and limit of quantification (LOQ) for all standards are presented in **Table II.1**.

3.1.3. Precision and accuracy

The method showed very good precision and accuracy. Repeatability, intermediate precision and accuracy are presented in **Table II.2**. These results suggested that the procedures described above were satisfactory with respect to both accuracy and precision. All precisions and accuracies results were acceptable.

Table II.1. Linearity, LOD and LOQ values for caffeic acid, *p*-coumaric acid, isoorientin and isovitexin.

Standard	Concentration range (μg mL^{-1})	Calibration curve	R^2	$F_{calculated}$*	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Caffeic acid	0.50 – 10.00	y = 9957547x – 1980541	1	1.37	0.65	1.97
***p*-Coumaric acid**	0.50 – 10.00	y = 65289976x + 522431	1	3.19	0.11	0.32
Isoorientin	7.81 – 250.00	y = 1939071x – 4248388	1	2.87	0.13	0.40
Isovitexin	7.81 – 250.00	y = 19173001x + 1904129	1	3.15	0.11	0.33

*$F_{calculated}$ should be lower than 3.26 ($F_{tabulated}$ at 95% with 4 and 12 degrees of freedom).

3.2 Method applicability: flavonoids quantification

The phenolic acids and flavonoids previously identified by HPLC-MS^n (Figueirinha *et al.*, 2008) were quantified by HPLC-DAD: 3-feroylquinic acid (**1**), neochlorogenic acid (**2**), chlorogenic acid (**3**), *p*-coumaric acid (**4**), carlinoside (**5**),

isoschaftoside (**6**), isoorientin (**7**), cynaroside (**8**), veronicastroside (**9**), luteolin 7-*O*-neohesperidoside (**10**), kurilensin A (**11**) and cassiaoccidentalin B (**12**). The chromatogram of CCI is representative of all three extracts and is depicted in **Figure II.1**. The concentration of each compound was determined by linear regression of the previously validated calibration curves and the results are expressed in the respective standard equivalents (**Table II.3**). The found amounts of each compound in the lemongrass extracts suggest that the most abundant phenolic compounds are 3-feroylquinic acid, chlorogenic acid, *p*-coumaric acid, isoorientin, kurilensin A and cynaroside. Moreover, all flavonoids are extracted in greater amounts when the polarity of the extraction solvent diminishes. These flavonoids have already been reported for their medicinal properties, such as antioxidant, anti-inflammatory and anticancer (Choi *et al.*, 2014; Hwang *et al.*, 2013; Kundu *et al.*, 2011; Park and Song, 2013; Yuan *et al.*, 2014). Nevertheless, carlinoside, cassiaocidentalin B and luteolin 7-*O*-neohesperidoside may also have some relevant roles in the therapeutic applications of lemongrass, since they have been described as anti-inflammatory, antioxidant and hepatoprotective (Abdel-Kader *et al.*, 2009; Pham *et al.*, 2013; Tomaino *et al.*, 2010).

Table II.2. Repeatability, intermediate precision and accuracy results for caffeic acid, *p*-coumaric acid, isoorientin and isovitexin.

Standard	Nominal concentration (μg mL^{-1})	Intra-day (n=3)			Inter-day (n=9)		
		Measured concentration (μg mL^{-1}) (mean ± SD)	Repeatability (% RSD)	Accuracy (% recovery)	Measured concentration (μg mL^{-1}) (mean ± SD)	Intermediate Precision (% RSD)	Accuracy (% recovery)
Caffeic acid	10.00	10.09 ± 0.02	3.44	100.93	9.85 ± 0.07	4.85	102.04
	2.50	2.72 ± 0.09	2.70	96.36	2.18 ± 0.10	4.80	98.73
	0.50	0.75 ± 0.06	3.20	95.65	0.61 ± 0.04	3.71	96.57
***p*-Coumaric acid**	10.00	9.89 ± 0.04	0.57	99.79	10.15 ± 0.03	0.47	100.65
	2.50	2.44 ± 0.06	0.75	100.46	2.58 ± 0.01	0.69	100.82
	0.50	0.65 ± 0.08	0.53	100.53	0.48 ± 0.07	0.43	101.26
Isoorientin	250.00	251.09 ± 0.02	2.71	100.57	249.15 ± 0.09	3.22	99.79
	62.50	61.02 ± 0.10	6.67	98.54	64.18 ± 0.12	6.18	103.78
	7.81	7.75 ± 0.07	6.92	110.96	7.86 ± 0.05	6.39	112.90
Isovitexin	250.00	252.17 ± 0.01	1.04	99.00	249.05 ± 0.07	0.75	98.61
	62.50	61.72 ± 0.08	0.98	103.17	63.08 ± 0.11	0.83	103.28
	7.81	7.92 ± 0.10	1.57	104.38	7.90 ± 0.04	1.33	104.22

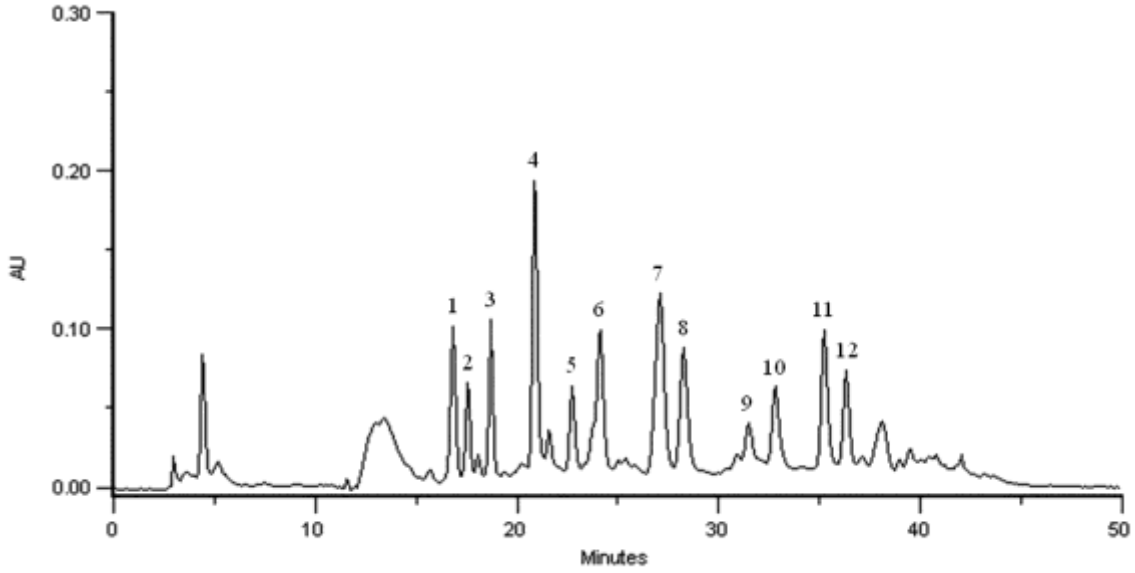

Figure II.1. HPLC-DAD profile of CCI (3mg mL^{-1}), recorded at 280 nm.

Table II.3. Phenolic compounds quantification by HPLC-DAD.

Peak	Compound	Measured concentration mean ± SD (μg mL^{-1})		
		CCI	CCM50	CCM100
1	3-Feroylquinic acid[a]	3.82 ± 0.04	3.75 ± 0.05	3.72 ± 0.08
2	Neochlorogenic acid[a]	2.16 ± 0.18	2.32 ± 0.12	2.46 ± 0.12
3	Chlorogenic acid[a]	4.40 ± 0.30	4.39 ± 0.14	4.53 ± 0.31
4	*p*-Coumaric acid[b]	8.01± 0.21	8.21 ± 0.05	8.12 ± 0.08
5	Carlinoside[c]	8.60 ± 0.11	16.30 ± 0.30	33.05 ± 0.17
6	Isoschaftoside[d]	11.35 ± 0.23	21.05 ± 0.14	45.06 ± 0.08
7	Isoorientin[c]	52.02 ± 0.54	100.14 ± 0.09	210.14 ± 0.27
8	Cynaroside[c]	23.45 ± 0.26	45.75 ± 0.19	87.09 ± 0.31
9	Veronicastroside[d]	3.26 ± 0.09	6.76 ± 0.28	11.96 ± 0.07
10	Luteolin 7-*O*-neohesperidoside[c]	11.12 ± 0.13	24.02 ± 0.17	43.72 ± 0.19
11	Kurilensin A[c]	26.48 ± 0.29	54.70 ± 0.05	104.48 ± 0.14
12	Cassiaoccidentalin B[c]	13.78 ± 0.16	27.06 ± 0.17	52.30 ± 0.18

[a]Concentration expressed in μg equivalents of caffeic acid per mL; [b]Concentration expressed in μg equivalents of *p*-coumaric acid per mL; [c]Concentration expressed in μg equivalents of isoorientin per mL; [d]Concentration expressed in μg equivalents of isovitexin per mL.

4. Conclusions

A simple and efficient reversed-phase HPLC-DAD method was successfully validated for simultaneous identification and quantification of phenolic compounds in *Cymbopogon citratus*, for the first time. The developed HPLC method was validated according to ICH and FDA guidelines. The method is simple, precise and accurate, presenting good LOD and LOQ values. It can be used for phenolic compounds quantification in other polar extracts and in different plants as well as in quality control of herbal formulations, with promising use in pharmaceutical and food supplement industries.

B. Influence of harvest time and material quality on phenolic content and antioxidant activity of *Cymbopogon citratus* infusion

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Abstract

Cymbopogon citratus (Cc) leaves are a product of high economic value because of its use in perfumery, cosmetic, pharmaceutical and agriculture industries. Polyphenols are most frequently present in combined form, and their structure and contents are often dependent of several factors like: plant varieties, culture conditions, maturation and processing. In this study, we aimed to evaluate the effect of the harvesting date and the quality of the plant material on the phenolic composition and the antioxidant activity of *C. citratus* leaves.

The plant material was classified accordingly to its harvest month for the same year: April to September and also accordingly to its quality (leaves ageing state): high, medium and low. A lipid-free infusion was prepared. Total hydroxycinnamic acids, flavonoids and tannins were evaluated by spectrophotometric methods and polyphenolic profile was attained by HPLC-DAD. Antioxidant capacity was assessed using DPPH, ABTS and FRAP assays.

Regarding the harvest date influence, total phenols were fairly constant from April to August, with a little decrease in September. Higher concentration of hydroxycinnamic acids was found in April and June, but much lower on the following months. With exception of August, almost the same percentage of flavonoids was registered for all months. On the other hand, we found that the best months for tannins biosynthesis were June, July and August. The samples collected in April and June were the most effective against all the oxidant species tested, suggesting a correlation between this parameter and amount of hydroxycinnamic acids.

In what concerns the contents of each polyphenol, evaluated by HPLC-DAD, some significant differences were observed for the hydroxycinnamic acids, which seem to be more influenced by the conditions studied (harvest month and material quality).

In conclusion, for *Cymbopogon citratus* leaves, both harvest date and plant quality are key criteria that have to be taken into account when selecting the material for human consuming, in order to receive benefits that have been

associated with phenolic compounds, namely to those who are present in this plant.

Keywords: *Cymbopogon citratus*; lemongrass; polyphenols; flavonoids; tannins; antioxidant; HPLC-DAD.

1. Introduction

Medicinal plants are commonly used to obtain phytotherapeutic medicines, food and cosmetics (Martinazzo *et al.*, 2009). However, their use is limited by several factors including: cultivation, harvest period, climatic factors, humidity, brightness, part of the plant, transportation method, storage, drying process and extraction process, which may all modify the composition of the products, directly affecting its safety and efficiency (Calixto, 2000).

Phenolic compounds are ubiquitous in the plant kingdom exerting several functions on plant. Many factors influence phenolic accumulation in plants although some are more significant than others. The hydric stress and other exogenous growth factors, exposure to various light sources and the presence of fungal or predatory pressures are shown to alter plant biosynthesis (Cohen and Kennedy, 2010). During plant growth, phenolic compounds protect fruits and vegetables from microbial damage, ultraviolet (UV) radiation, and predation among other environmental stresses. The sun exposure is credited with being a major element relating to plant metabolism. It is now a well-established knowledge that sunlight itself directly influence the phenolic compounds biosynthesis (Zucker, 1965). The major challenge on studying sun exposure and plants is the dual effect of radiation. As the amount of sun exposure increases there is a concomitant rise in temperature (Spayd *et al.*, 2002). Both flavonoids and proanthocyanidins showed significant increases with sun exposure (Spayd *et al.*, 2002). Also phenolic acids are affected by UV radiation (Booij-James *et al.*, 2000). As seen in sun exposure, temperature can also be correlated to phenolic composition (e.g. anthocyanins) but breaching a critical limit (high and low) has detrimental effects. It is likely that all plants exhibit temperature thresholds (Bradfield and Stamp, 2004). Furthermore, the phenolic composition, the degree of hydroxylation and acylation, is also sensitive to temperature shifts and likely reflects different sensitivity of various genes or enzymes in the flavonoid pathway (Cohen *et al.*, 2008). The phase of postharvest, more precisely the drying process, requires special attention; therefore, the choice of dryer, drying system, air composition, height of layers of leaves in the dryer, and temperature and relative

humidity of the room air can provide plants with better quality after harvesting. The rate at which water is removed from the medicinal plant during drying is very important because a rapid process can degrade active ingredients (Coradi *et al.*, 2014).

Cymbopogon citratus (DC.) Stapf, Poaceae, is an aromatic herb known as lemongrass, native from India and tropical Asia and currently it grows worldwide. Lemongrass is a crop of high economic value because of its use in the perfumery, cosmetic, pharmaceutical and food industries (Oyedele *et al.*, 2002; Saddiq and Khayyat, 2010). *C. citratus* is commercially cultivated in the Democratic Republic of Congo, Angola, Madagascar and Comoros Island. However, the leading exporter of this plant is Guatemala, trading about 250,000 kg per year, while the USA imports about 70,000 kg per year (Department of Agriculture, Forestry and Fisheries, 2012). The commercial value of lemongrass species is further enhanced by their ability to grow in moderate and extremely harsh climatic conditions (Padalia *et al.*, 2011). *C. citratus* is commonly used in folk medicine for treatment of nervous and gastrointestinal disturbances, and as antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic and sedative (Santin *et al.*, 2009). Recently, our group has been involved in the identification of functional compounds from lemongrass. We demonstrated that flavonoids, such as luteolin and apigenin glycosides, and proanthocyanidins strongly contributed to antioxidant and anti-inflammatory properties of an essential oil-free infusion from lemongrass (Costa *et al.*, 2015a; Figueirinha *et al.*, 2010, 2008; Francisco *et al.*, 2014, 2013, 2011).

Factors such as harvest season and quality grades are usually not taken into account for this plant, since it is harvest along the whole year, except for the winter (Department of Agriculture, Forestry and Fisheries, 2012). Hence, the chemical composition of the plant may vary from one month to another, leading to different industrial final products. Quality grading, performed by expert graders from industry, entails evaluating the appearance of the fresh and/or dry leaves. A literature search was undertaken on the effect of different methods of drying on chemical composition and content of the essential oil. The results showed that drying method had a significant effect on oil content and composition of aromatic

plants (Okoh *et al.*, 2008). However, to our knowledge, there are no reports of those effects on the polyphenolic content of lemongrass.

Therefore, the aim of this study was to investigate the effect of the harvest time and plant quality on the qualitative and quantitative phenolic composition of *C. citratus* infusion.

2. Materials and Methods

2.1 Plant material

Dry leaves of *Cymbopogon citratus* were acquired from ERVITAL (Mezio, Castro Daire, Portugal). *Cymbopogon citratus* was bred in the region of Mezio, Castro Daire (Portugal), located in a greenhouse located 1000 m above sea level. A voucher specimen is 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 (Life Sciences Department, University of Coimbra, Portugal). The plant material was harvest within the year of 2011, and classified accordingly to two parameters: its harvest date - April (Apr), June (Jun), July (Jul), August (Aug) and September (Sep) - and its quality grade, by leaves ageing state - high (H), medium (M) and low (L) quality, where high quality corresponds to young fully-grown green leaves and low quality corresponds to aged brownish leaves. This criterion to classify the plant material according to its quality was set by the producer. A lipid- and essential oil-free infusion (extract) was prepared from each sample as previously described (Figueirinha *et al.*, 2008). Briefly, the infusion was prepared by adding boiling water to the plant material, the mixture being kept hot and left to stand for 15 min. The extract was then filtered under vacuum, concentrated and freeze-dried. The freeze-dried extract was kept at -20ºC until use.

2.2 Polyphenols quantification

2.2.1. Total phenols

A modified version of the Folin-Ciocalteu method (Wang, C., Lee, W., Peng *et al.*, 1997) was used. Briefly, the sample (100 μL) was mixed with 1 mL of Folin-

Ciocalteau reagent (Merck®, Darmstadt, Germany) and 5 mL of sodium carbonate (20%, w/v) (Merck®, Darmstadt, Germany), and the final volume was completed with MilliQ water. The absorbance was measured at 700 and 735 nm and the results expressed as grams of gallic acid equivalents by 100 g freeze-dried extracts. The extracts of all samples were analyzed in triplicate.

2.2.2. Total hydroxycinnamic acids

The method described by Lamaison and co-workers (Lamaison *et al.*, 1991) was used. Briefly, Arnow reagent (1 mL) was added to the extracts in 50% ethanol (1 mL), previously mixed with HCl 0.5N (1 mL). Then, 1 mL of NaOH 1N was added to the mixture and the final volume was completed with MilliQ water. The absorbance was measured immediately at 505 nm, and the results were expressed in grams caffeic acid equivalents by 100 g of freeze-dried extracts. The extracts of all samples were analyzed in triplicate.

2.2.3. Total flavonoids

Reagent [$AlCl_3 \cdot 6H_2O$ 2% in methanol (w/v), 2 mL] was added to the extracts in ethanol (2 mL). The absorbance was measured after 10 min, at 430 nm (Lamaison and Carnat, 1990), and the results were expressed in grams rutin equivalents by 100 g of freeze-dried extracts. The extracts of all samples were analyzed in triplicate.

2.2.4. Total tannins

The European Pharmacopeia official method was used. Briefly, the extracts were solubilized in MilliQ water (500 mg / 2 mL), and then mixed with 1 mL of Folin-Ciocalteau reagent (Merck®, Darmstadt, Germany) and 10 mL of MilliQ water, and the final volume was completed with sodium carbonate (20%, w/v) (Merck®, Darmstadt, Germany). The same procedure was applied to the extract solutions, previously treated with skin powder (1%, w/v) for 1 hour. The absorbance of both solutions was measured at 760 nm after 30 min, and the

results expressed as grams of pyrogallol equivalents by 100 g freeze-dried extracts. The extracts of all samples were analyzed in triplicate.

2.2.5. HPLC-DAD analyses

HPLC analyses were performed in a chromatograph GILSON equipped with a diode-array detector (DAD) (Gilson® Electronics SA, Villiers le Bel, France). The studies were carried out on a Spherisorb S5 ODS-2 column (250 x 4.6 mm i.d.; particle size, 5 μm; Waters® Corp., Milford, MA, USA) and a Nucleosil C18 guard cartridge (30 x 4 mm i.d.; particle size, 5 μm; Macherey-Nagel, Düren, Germany) at 25ºC. A mobile phase of 5% (v/v) aqueous formic acid (A) and methanol (B) was used with a discontinuous gradient: 5–15% B (0–10 min.), 15–30% B (10–15 min.), 30–35% B (15–25 min.), 35–50% B (25–35 min.) and 50–80% B (35–40 min.), followed by an isocratic elution for 20 min., at a flow rate of 1 mL/min. An injection volume of 100 μL was used for all standards and samples. Chromatographic profiles were acquired in the wavelength range 200–600 nm, and recorded at 280 nm. Data treatment was carried out with Unipoint® 2.10 software (Gilson®). For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (0.25–10 μg/mL for caffeic acid and *p*-coumaric acid; 0.08-3.30μg/mL for isovitexin and isoorientin) of the different standards: caffeic acid ($y = 9957547x - 1980541$; $R^2 = 0.9978$); *p*-coumaric acid ($y = 65289976x + 522431$; $R^2 = 0.9999$); isovitexin ($y = 19173001x + 1904129$; $R^2 = 0.9994$); isoorientin ($y = 11857477x + 1553624$; $R^2 = 0.9992$). The results were expressed in percentage, g of respective standard equivalent per 100 g of extract, as mean ± standard deviation of three independent analyses.

2.3 Antioxidant activity

2.3.1. DPPH radical assay

Free radical-scavenging activity was evaluated according to the method described by Blois and colleagues (Blois, 1958). Aliquots of extract methanolic solution (100 μL) were assessed by their reactivity with a methanolic solution of

500 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH) (500 μL) (Sigma-Aldrich, Química S.A., Portugal) in the presence of 100 mM acetate buffer, pH=6.0 (1 mL). Reaction mixtures (3 mL) were kept for 30 min at room temperature and in the dark. The decreases in the absorbance were measured at 517 nm. Different dilutions of each of the test compounds were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox® (62.5-1000 μM) (Sigma-Aldrich, Química S.A., Portugal). Two independent experiments in triplicate were performed for each extract. Results were expressed as Trolox® equivalent antioxidant capacity (TEAC), defined as the concentration of the extract whose antioxidant capacity is equivalent to a 1.0 mM of Trolox® (Antolovich *et al.*, 2002).

2.3.2. ABTS (pH=4) radical assay

The assay was carried out according to Cano and co-workers (Cano *et al.*, 1998) with minor modifications made by Villaño and colleagues (Villaño *et al.*, 2004). Free radicals were generated by an enzymatic system consisting of horseradish peroxidase enzyme, its oxidant substrate (hydrogen peroxide) and the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) chromophore. The radical was generated by a reaction between 1.5 mM ABTS (Sigma-Aldrich, Spain), 15 μM hydrogen peroxide (Sigma-Aldrich, Spain) and 0.25 μM peroxidase (Sigma-Aldrich, Spain) in 50 mM glycine-HCl buffer (pH=4). The final volume was 60 mL, yielding a final concentration of 30 μM of the $ABTS^{\bullet+}$ radical cation. The blank reference cuvette contained glycine–HCl buffer. Once the radical was formed, the extract was added and the decrease in absorbance was evaluated. The assay was carried out at room temperature. The reaction started by adding 100 μL of test extract to 2 mL of $ABTS^{\bullet+}$ solution, the mixture was vortexed for 10 s, and the absorbance at 414 nm was measured after 2 min of reaction. Two independent experiments in triplicate were performed for each extract, six different dilutions being prepared in 50% aqueous methanol and submitted to the reaction. TEAC values were obtained by interpolating the decrease in absorbance on the calibration curve obtained using Trolox® solutions from 62.5 to 500 μM.

2.3.3. ABTS (pH=7) radical assay

In this assay, the $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulphate (2.45 mM, final concentration) (Merck, Darmstadt, Germany) in water. The mixture was allowed to stand in the dark at room temperature for 12–16 h before use, and then the $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS) at pH=7 and equilibrated at 30 ºC to give an absorbance of 0.7±0.02 at 734 nm. Aliquots (50 μL) of 50% aqueous methanol of the test extract were mixed with 2 mL of the $ABTS^{\bullet+}$ preparation, vortexed for 10 s, and the absorbance measured at 734 nm after 4 min of reaction at 30 ºC. Different dilutions of each extract were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox® (62.5–500 μM). The results were expressed as TEAC values. Two independent experiments in triplicate were performed for each extract.

2.3.4. Ferric reducing power assay

Ferric reducing ability was evaluated according to Benzie and Strain (Benzie and Strain, 1996) with minor modifications. The FRAP reagent contained 10 mM of TPTZ (Sigma-Aldrich, Spain) solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ (Merck, Darmstadt, Germany) and acetate buffer (300 mM, pH=3.6) (1:1:10, v/v/v). Aliquots (100 μL) of 50% aqueous methanol of the test extract were added to 3 mL of the FRAP reagent, and the absorbance was measured at 593 nm after incubation at room temperature for 6 min, using the FRAP reagent as blank. Different dilutions of each extract were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox® (31.25-1000 μM). The results were expressed as TEAC values. Two independent experiments in triplicate were performed for each extract.

3. Results

3.1 Polyphenols quantification

3.1.1. Evaluation by spectroscopic methods

Figure II.2 displays the amount of total phenols, hydroxycinnamic acids, flavonoids and tannins in each harvest moth. For this assay only high-quality samples were used. Total phenols contents seem to be quite stable in April (5.67%), June (5.55%) and July (5.35%), followed by an increase in August (6.14%) and a decrease to minimum levels in September (4.58%). The hydroxycinnamic acids present their maximum values in April (4.96%) and June (5.08%), but suffer a 2% fall in the following months. Total flavonoids were the phenolic compounds which have the most significant decrease in a given harvest date, August (4.81%), exhibiting their maximum values in June (6.63%) and September (6.62%). Total tannins increase from April (2.89%) to August (3.96%), followed by a decrease to minimum levels in September (2.97%).

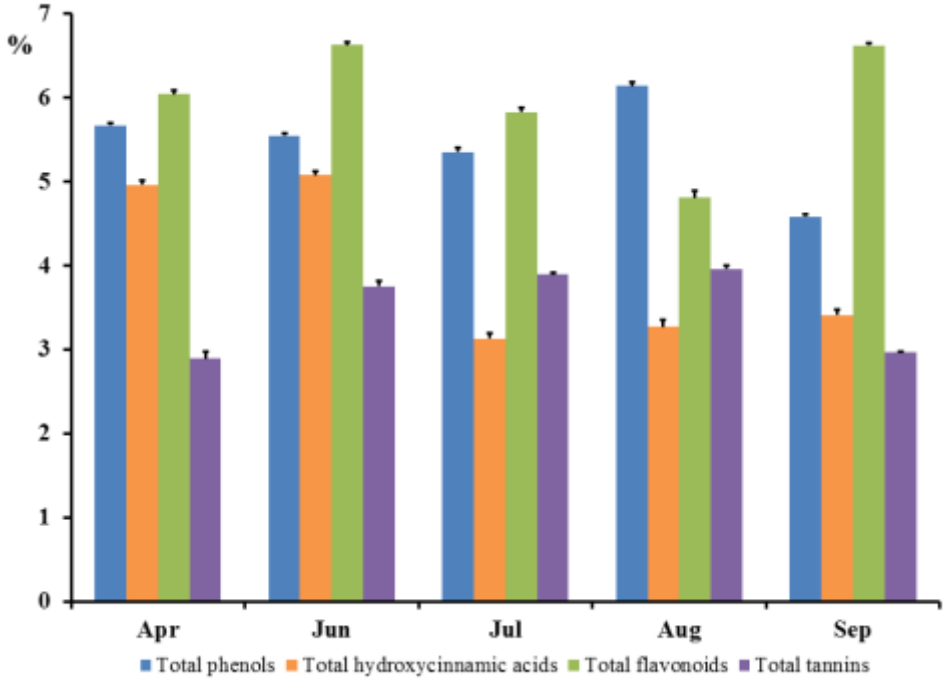

Figure II.2. Phenolic compounds amount (%, g of respective standard equivalents per 100 g of extract) quantified in lemongrass infusion of samples harvested from April to September.

Figure II.3 shows the phenolic content of four lemongrass samples: high- and low-quality, harvested in April; and medium- and high-quality, harvested in June. In all studied samples the amount of all groups of phenolic compounds is higher in high-quality samples and lower in low-quality samples. A correlation between phenolic compounds content and sample quality was markedly observed.

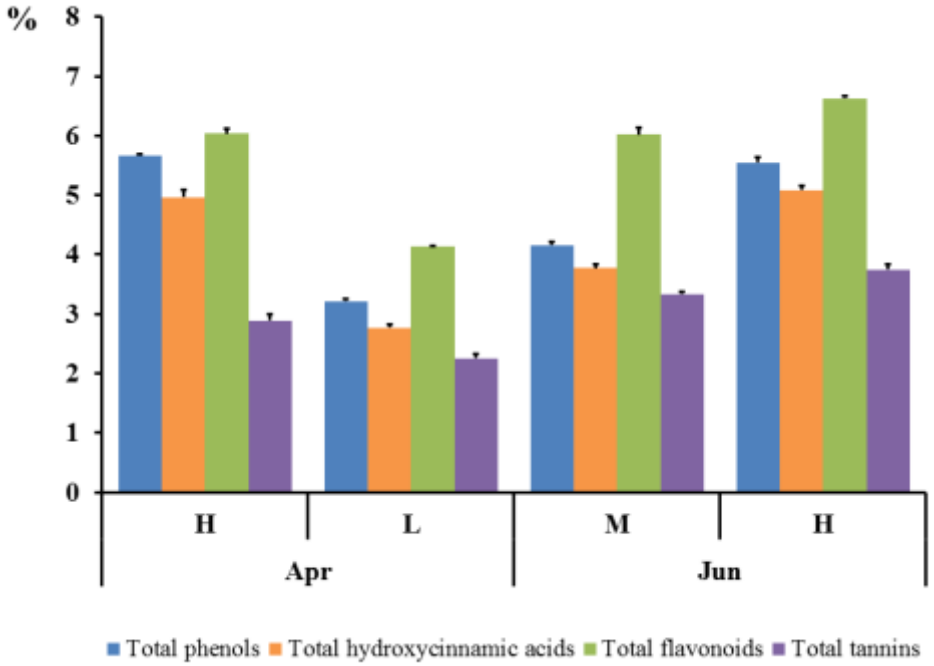

Figure II.3. Phenolic compounds amount (%, g of respective standard equivalents per 100 g of extract) quantified in lemongrass infusion of high-quality (H) and low-quality (L) samples harvested in April, and in the infusion of medium-quality (M) and high-quality (H) samples harvest in June.

3.1.2 HPLC-DAD analyses

HPLC-DAD phenolic profiles from *C. citratus* infusions were very similar for all analysed samples, exhibiting only slight quantitative differences. The chromatographic profile of high-quality April-harvested lemongrass infusion was found to be representative of all samples (**Figure II.4**). The following compounds were identified: 3-*O*-feruloylquinic acid (**1**), 5-*O*-caffeoylquinic acid (neochlorogenic acid) (**2**), 3-*O*-caffeoylquinic acid (chlorogenic acid) (**3**), *p*-coumaric acid (**4**), luteolin 6-*C*-glucosyl-8-*C*-arabinoside (carlinoside) (**5**), apigenin 6-*C*-arabinosyl-8-*C*-glucoside (isoschaftoside) (**6**), luteolin 6-*C*-glucoside (isoorientin) (**7**), isoorientin 2''-*O*-rhamnoside (**8**), luteolin 7-*O*-neohesperidoside

(**9**), luteolin 6-*C*-arabinosyl-2''-*O*-rhamnoside (kurilensin A) (**10**), luteolin 6-*C*-pentoside (**11**) and luteolin 2″-*O*-rhamnosyl-6-*C*-(6-deoxy-ribo-hexos-3-ulosyl) (cassiaoccidentalin B) (**12**). Compounds identification was achieved by HPLC-PDA-ESI/MS^n as previously described (Figueirinha *et al.*, 2008; Francisco *et al.*, 2014; Tavares *et al.*, 2015).

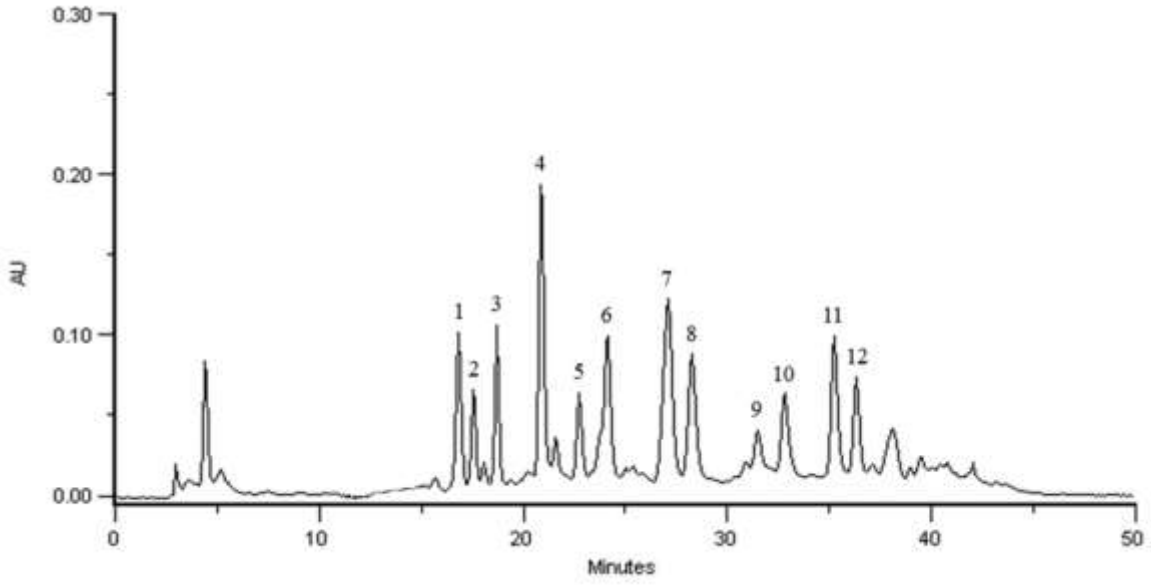

Figure II.4. HPLC-DAD profile of high quality lemongrass infusion (harvested in April), recorded at 280 nm.

In **Figure II.5** and **Table II.4**, it may be observed the percentage variation of each compound identified in lemongrass infusion over spring-summer months. Only high-quality samples were used. Hydroxycinnamic acids seem to be the group of compounds that suffers the most variability. Compound **1** increased from April (0.11%) to September (0.19%), with a smooth breakdown in July (0.14%). Compound **2** exhibited its highest amount in June (0.22%), followed by a substantial breakdown in the rest of the summer. Compound **3** showed a consistent increase over all tested months, exhibiting its highest amount in September (0.16%). Compound **4** kept almost the same percentage over time (0.03-0.04%).

In what concern the quantified flavonoids, there were no significant changes on all months tested, with the exception of compound **7**, which exhibited its

maximum amount in April (0.13%), and then a constant decrease in the following months until reaching a stable plateau in August and September (0.09%).

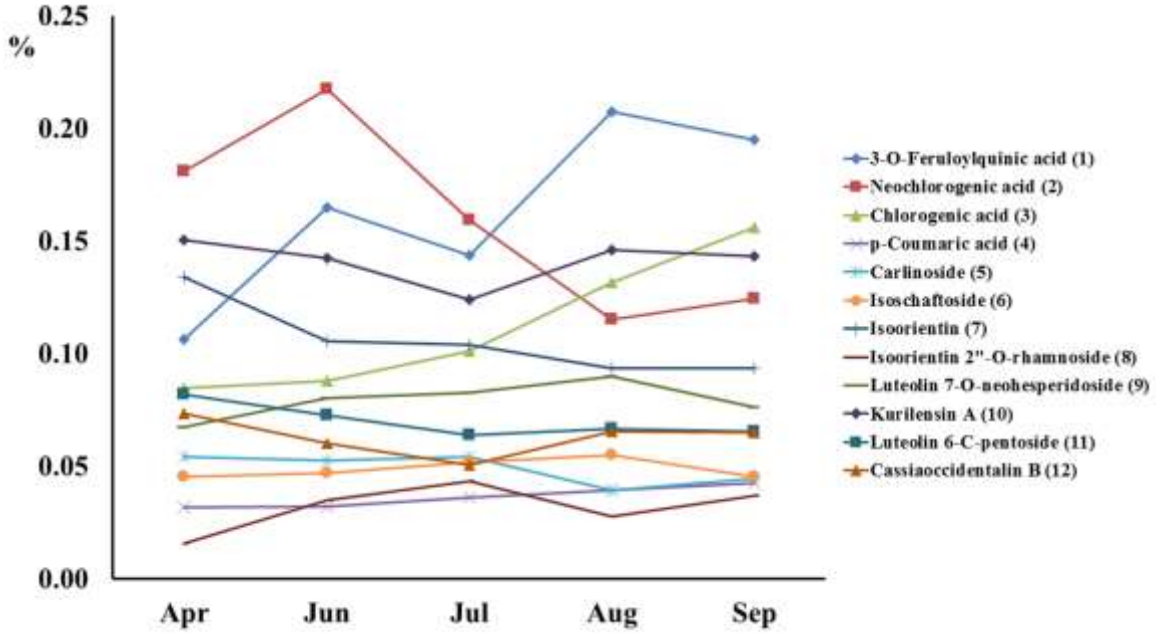

Figure II.5. Phenolic compounds (**1-12**) quantified by HPLC-DAD in lemongrass infusion of samples harvested from April to September. The results are expressed in percentage, g of respective standard equivalent per 100 g of extract (compounds **1-3** in caffeic acid equivalents; compound **4** in *p*-coumaric acid; compounds **5** and **7-12** in isoorientin equivalents; compound 6 in isovitexin equivalents).

Table II.4. Phenolic compounds (**1-12**) quantified by HPLC-DAD in lemongrass infusion of samples harvested from April to September. The results are expressed in percentage, g of respective standard equivalent per 100 g of extract (mean ± SEM).

Compound	April	June	July	August	September
3-*O*-Feruloylquinic acid (**1**)	0.11 ± 0.01	0.16 ± 0.02	0.14 ± 0.01	0.21 ± 0.08	0.19 ± 0.01
Neochlorogenic acid (**2**)	0.18 ± 0.02	0.22 ± 0.07	0.16 ± 0.05	0.12 ± 0.04	0.12 ± 0.02
Chlorogenic acid (**3**)	0.08 ± 0.03	0.09 ± 0.02	0.10 ± 0.05	0.13 ± 0.08	0.16 ± 0.03
p-Coumaric acid (**4**)	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.01
Carlinoside (**5**)	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.02
Isoschaftoside (**6**)	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.03	0.05 ± 0.03
Isoorientin (**7**)	0.13 ± 0.05	0.11 ± 0.01	0.10 ± 0.06	0.09 ± 0.02	0.09 ± 0.02
Isoorientin 2"-*O*-rhamnoside (**8**)	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
Luteolin 7-*O*-neohesperidoside (**9**)	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	0.09 ± 0.02	0.08 ± 0.02
Kurilensin A (**10**)	0.15 ± 0.03	0.14 ± 0.05	0.12 ± 0.09	0.15 ± 0.07	0.14 ± 0.01
Luteolin 6-*C*-pentoside (**11**)	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.03	0.07 ± 0.02	0.07 ± 0.01
Cassiaoccidentalin B (**12**)	0.07 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	0.07 ± 0.01	0.07 ± 0.02

Figure II.6 illustrates the amount of each quantified phenolic compound in lemongrass infusion, extracted from four lemongrass samples: high- and low-quality, harvested in April; and medium- and high-quality, harvested in June. As observed for the quantified phenolic compounds (see section **3.1.1**), all detected polyphenols decrease with the degradation of the vegetable material, presenting their maximum values in high-quality samples and their minimum percentages in the low-quality sample.

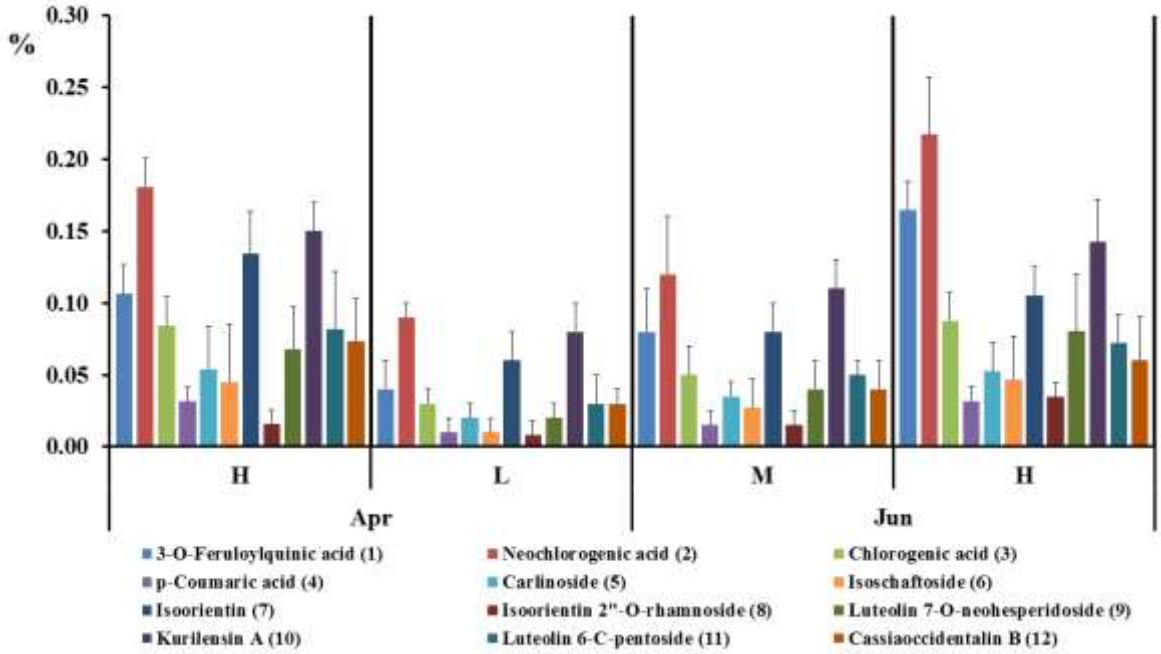

Figure II.6. Phenolic compounds (**1-12**) quantified by HPLC-DAD in lemongrass infusion of high-quality (H) and low-quality (L) samples harvested in April, and in the infusion of medium-quality (M) and high-quality (H) samples harvest in June. The results are expressed in percentage, g of respective standard equivalent per 100 g of extract.

3.2 Antioxidant activity

Figure II.7 displays the antioxidant capacity against the different tested oxidant species for each harvest moth. Only high-quality samples were used. Both DPPH, ABTS (pH=7) and FRAP assays showed that the plant extracts possess very similar antioxidant activities over the months, with a swift decrease in September. In contrast, the tested samples from April to July exhibited a drastic decrease in the antiradical effect for the ABTS (pH=4) assay, maintaining the antioxidant levels afterwards. Furthermore, the present data show that the

lemongrass infusion is more effective against ABTS (pH=7) and DPPH, in comparison against the other evaluated species.

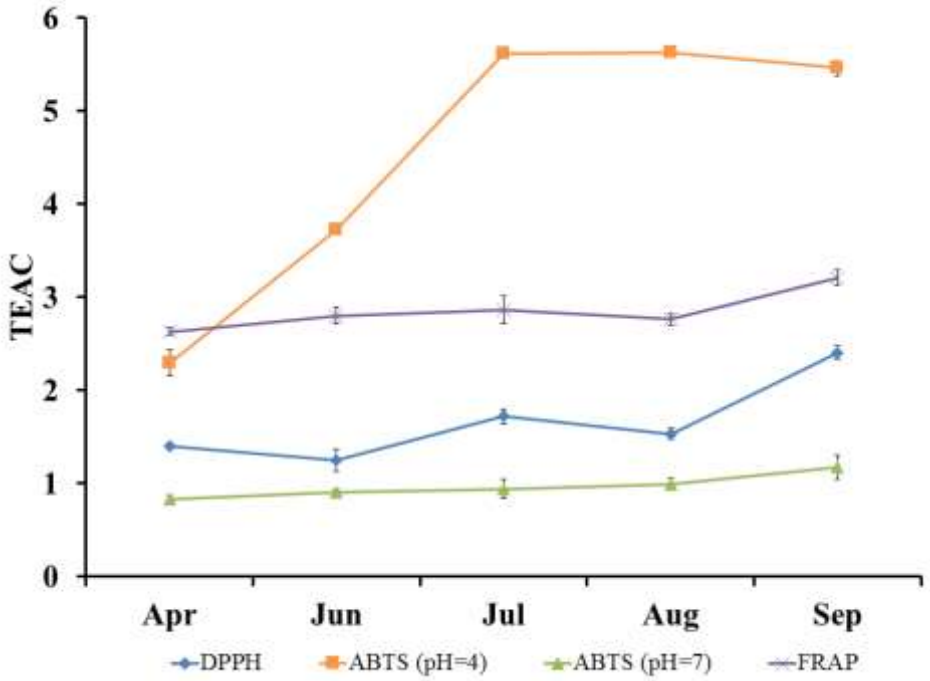

Figure II.7. Antioxidant activity (expressed in TEAC value) of lemongrass extracts harvested from April to September.

Figure II.8 shows the antioxidant capacity against the different tested oxidant species of samples with distinct quality levels. In all studied samples the antioxidant activity against all tested oxidant species is directly related to the quality level.

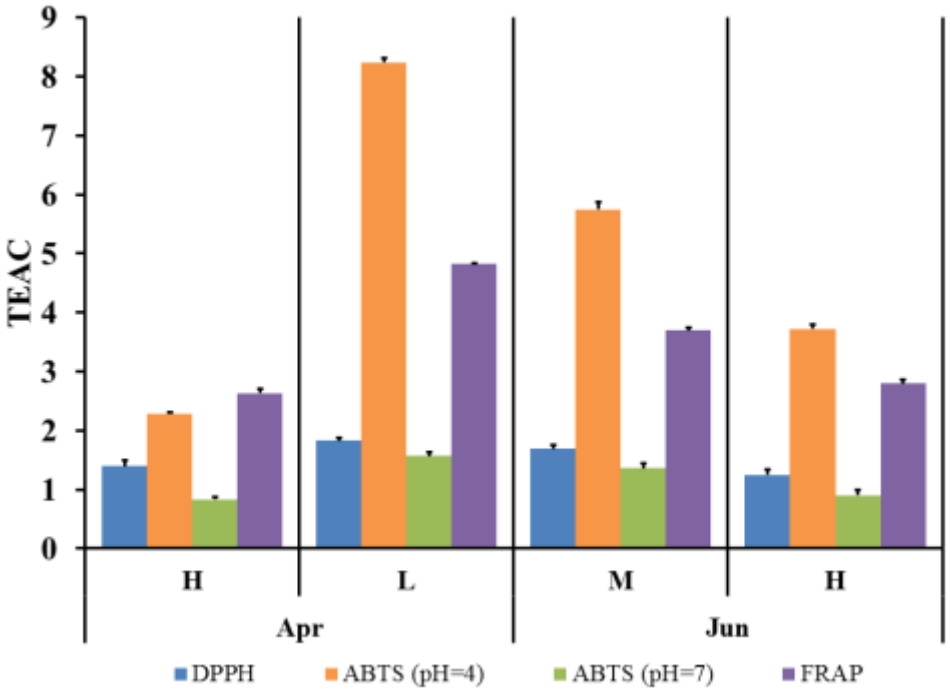

Figure II.8. Antioxidant activity (expressed in TEAC value) of lemongrass extracts from high-quality (H) and low-quality (L) samples harvested in April, and of medium-quality (M) and high-quality (H) samples harvest in June.

4. Discussion

Results obtained in this work suggest that phenolic compounds contents can be related to the harvest date. The total phenols assay showed a substantial loss from August to September, suggesting that the biosynthesis of these compounds is affected by the sunlight, such was suggested by Eichholz *et al.* (2011) and Namlı *et al.* (2014) in *Vaccinium corymbosum* and *Hypericum retusum*, respectively, or temperature, decreasing with the arrival of autumn. In contrast, the total flavonoids show a great increase in the same period. Therefore, the climatic conditions such as sunlight intensity and period, temperature, air composition and wind dynamics, during that time of the year seem to favour the biosynthesis of these phenolic compounds, as referred by other authors (Martínez-Lüscher *et al.*, 2014; Vidović *et al.*, 2015). Hydroxycinnamic acids, another relevant group of phenolic compounds on lemongrass infusion, are more affected by the beginning of the summer, as observed in the remarkable breakdown from June to July.

Nevertheless, the best month to harvest lemongrass depends on which group of phenolic compounds is more relevant for its use. For tannins, summer months (June, July and August) must be selected. In what concern hydroxycinnamic acids, they are more abundant in April and June. Flavonoids should be better extracted from plant material collected either in June or in September. This data is also extremely important if one wants to take advantage of a biological beneficial effect of a single phenolic compounds group. For instance, lemongrass hydroxycinnamic acids and flavonoids have showed to possess a significant anti-inflammatory potential (Figueirinha *et al.*, 2010, 2008; Francisco *et al.*, 2014, 2013, 2011), while tannins have a greater effect against oxidant species (Costa *et al.*, 2015a).

Regarding the influence of plant quality on the composition of lemongrass extract, it is clear the correlation between these two parameters. Regardless the group of phenolic compound addressed, its content was always inversely proportional to the degree of leaves ageing, and therefore, to the time of sunlight and/or UV light exposition. These data are corroborated by other authors (Joubert *et al.*, 2012; Stanimirova *et al.*, 2013). Nevertheless, a recent work proved that UV-B supplementation, in low doses at physiological conditions, increases the amount of phenolic compounds biosynthesized by this plant (Kumari and Agrawal, 2010).

Antioxidant assays showed that all samples exhibited reasonably good TEAC values, with the exception for the ABTS (pH=4) assay. In that case, lemongrass infusion anti-radical power is tightly dependent on the harvest month, since it decreases drastically from April to July. This radical at pH=4 could be a significant marker for antioxidant activity monitoring. For other three oxidant species tested, the relationship between the harvest date and antioxidant potential is not very straight, with the exception of the sample collected in September, to which the antioxidant capacity decreased slightly. Nevertheless, it is clear the influence of harvest season on the antioxidant activity of lemongrass, as described by Zhu and co-workers for the phenolic acids and flavonoids from blueberries (*Vaccinium ashei*) (Zhu *et al.*, 2013). Among the different groups of phenolic compounds, the phenolic acids seem to be the most decisive in the overall antioxidant activity of

the extract. For instance, in April and June, which are the months when the highest amount of phenolic acids is synthesized, the antioxidant potential is at its highest. Curiously, this data is corroborated by a recent review (Razzaghi-Asl *et al.*, 2013).

In what concerns the quality of the harvested material and its antioxidant capacity, the relationship could not be more explicit. For all tested oxidant species, the high-quality samples exhibited the best antioxidant results, and samples collected at the same month, but with low-quality presented the poorest potential. These results are corroborated by recent works (Klavsen and Madsen, 2008; Nantitanon *et al.*, 2010). The difference is more pronounced for ABTS radical (at pH=4) and for ferric reducing power. On the other hand, all samples showed a better activity against ABTS (at pH=7) and DPPH radicals.

The phenolic compounds quantification allowed investigating the variation of each particular phenolic compound for the different types of samples. Ferulic and caffeic acid derivatives (**1-3**) seem to be more sensitive to environment changes. These findings agree with those reported by Kotilainen and colleagues (Kotilainen *et al.*, 2008), in which a decrease in some phenolic acids occurred upon irradiance with UV-B or UV-B + UV-A. On the other hand, *p*-coumaric acid (**4**) showed no substantial differences in the period of time tested, as described by Agati and co-workers in *Ligustrum vulgare* (Agati *et al.*, 2011). Flavonoids seem to be more stable on their individual amounts, exception made to isoorientin. This flavonoid, besides being the most abundant flavonoid in lemongrass infusion, is also the most variable through the harvesting season. This point is crucial to consider when the bioactivity of this compound is at stake. In fact, isoorientin has been associated with significant biological activities such as antioxidant and anti-inflammatory (Francisco *et al.*, 2014; Orrego *et al.*, 2009). Several authors suggest that this particular group of phenolic compounds act as a response to UV stress, that is regarded as an oxidative stress, and also as "sunscreen compounds" aiming at countering the UV-B-induced oxidative damage (Agati *et al.*, 2011; Xu *et al.*, 2008). Moreover, increased UV-B induces the rapid synthesis of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) to cope with the free superoxide radicals. It is supposed that peroxidases under UV-B stress can

use flavonoids as substrates to detoxify hydrogen peroxide (Zlatev *et al.*, 2012). The classical model proposed by Yamasaki *et al.* (1997), in which B-ring *ortho*-dihydroxylated flavonoids are effective substrates for class III peroxidases to reducing H_2O_2 may be corroborated in light of the data presented here, since this specific type of flavonoids are the most abundant in lemongrass infusion.

The effect of material quality on the amount of each phenolic compound was again disclosed. Once again, the percentage of all compounds is greater as the quality of the harvested vegetable material increases.

5. Conclusions

Our results indicate that the content on polyphenols and the antioxidant capacity of *Cymbopogon citratus* infusion is strongly related with the quality of the plant at the moment it is harvested. The effects of UV irradiance on the biosynthesis of flavonoids with potentially effective antioxidant activities lead to the hypothesis that light-induced changes in flavonoid metabolism are aimed at equipping the leaves with effective antioxidant metabolites, namely tannins. Therefore, it is not indifferent to extract the phenolic compounds from this plant with different quality grades or harvested on different months of the year. However, antioxidant activity and flavonoid concentrations were evaluated only at five consecutive months and with only one medium-quality and one low-quality samples, making it difficult to correlate the extent of environmental factors with polyphenolic content. This interesting issue may merit further investigations in which the composition on all phenolic compounds and in every month of the year are studied.

In conclusion, both harvest date and plant quality are key criteria that have to be taken into account when selecting the material for human consuming, in order to receive benefits that have been allocated to phenolic compounds, namely to those who are present in this plant.

C. Flavan hetero-dimers in the *Cymbopogon citratus* infusion: tannin fraction obtaining, evaluation of its contribution to the antioxidant activity and structural elucidation

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Abstract

Cymbopogon citratus (lemongrass) leaves infusion, a commonly used ingredient in Asian, African and Latin America cuisines, is also used in traditional medicine for the treatment of several pathologic conditions, however little is known about their bioactive compounds. Recent studies revealed the crucial role of the phenolic compounds on the infusion bioactivity, namely flavonoids and tannins. Flavonoids have already been characterized; however the tannin fraction of lemongrass infusion is still uncharted. The aim of the present work is to characterize this fraction, and to evaluate its contribution to the antioxidant potential of this plant. Chemical characterization was achieved by HPLC-DAD-ESI/tandem MS and antioxidant activity was evaluated using DPPH, ABTS and FRAP assays. Hetero-dimeric flavan structures have been described for the first time in lemongrass consisting of apigeniflavan or luteoliflavan units linked to a flavanone, either naringenin or eriodictyol, which may occur as aglycone or glycosylated forms. The antioxidant capacity of the fraction containing these compounds was significantly higher than the infusion, indicating its potential as a source of natural antioxidants.

Keywords: *Cymbopogon citratus*; lemongrass; Poaceae; polyflavans; polyphenols; tannins; antioxidant.

1. Introduction

Cymbopogon citratus (DC.) Stapf, commonly known as lemongrass, belongs to the Poaceae family and is a tropical perennial shrub originated from Southeast Asia. The lemon-like flavour of the plant is responsible for its use in tropical countries cuisines being its leaves a common ingredient in Asian cuisine in teas, soups and curries, being also suitable for fish, seafood and poultry (Adeneye and Agbaje, 2007). In African and Latin American countries, this herb is highly consumed as an aromatic and pleasant-tasting herbal drink. Furthermore, this plant is reported to possess antifungal, mosquito repellent, insecticidal, anti-diabetic, anti-septic, anti-mutagenic and anti-carcinogenic activity. The tea prepared from its leaves is medicinally used in Japan and Brazil (Masuda *et al.*, 2008), and aqueous extracts of dried leaves are used in folk medicine for the treatment of several inflammation-based pathologies (Shah *et al.*, 2011). The antioxidant and radical scavenging activities of hydrophilic extracts of *Cymbopogon citratus* have been reported by several authors and related to its polyphenolic components (Cheel *et al.*, 2005; Khadri *et al.*, 2008; Yoo *et al.*, 2008). A recent work also revealed the high capacity of an aqueous extract from lemongrass to protect against the hydrogen peroxide-induced oxidative stress (Rahim *et al.*, 2013).

Previous studies suggested that lemongrass contains tannins, namely proanthocyanidins, based on the observation that acid cleavage of a fraction isolated from its lipid-free infusion yielded anthocyanidin-type products, although the precise nature of the compounds was not established (Figueirinha *et al.*, 2010). Proanthocyanidins are oligomeric and polymeric flavonoids composed of flavan-3-ol subunits linked by C-C bonds. They are widespread throughout the plant kingdom, where they accumulate in many different organs and tissues, providing protection (Quijada-Morín *et al.*, 2012). Much of the earlier research on proanthocyanidins refers to flavan-3-ol and flavan-3,4-diols oligo/polymers, although other structures able to release anthocyanidins upon heating in acidic alcohol solutions have also

been described. Thus, in sorghum, also belonging to the Poaceae family, Gujer and co-workers (Gujer *et al.*, 1986) identified unique hetero-dimers and trimers consisting of a flavanone, either eriodictyol or eriodictyol 5-*O*-glucoside, as the terminal unit linked to one or two glucosylated luteoliflavan (i.e. 5,7,3',4'-tetrahydroxyflavan-5-*O*-glucosyl) extending units. The presence in sorghum of that type of oligomers containing either luteoliflavan or apigeniflavan extending units (i.e. releasing the 3-deoxyanthocyanidins luteoliflavan and apigeniflavan upon acid cleavage) up to the heptamer was further confirmed by Krueger and colleagues (Krueger *et al.*, 2003).

The present work aims to characterize phytoconstituents of the tannin fraction from *Cymbopogon citratus* infusion, and to evaluate its contribution to the antioxidant potential of this plant.

2. Materials and Methods

2.1 Plant material, extract preparation and fractioning

Dry leaves of *Cymbopogon citratus* were purchased from ERVITAL (Mezio, Castro Daire, Portugal). The plant was cultivated in the region of Mezio, Castro Daire (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 (Life Sciences Department, University of Coimbra, Portugal). A lipid- and essential oil-free infusion was prepared (CcI) as previously described (Figueirinha *et al.*, 2008). Afterwards, the extract was fractionated on a reverse phase preparative Flash Chromatography® C18 column Buchi® (150 × 40 mm; particle size 40 - 63 μm) (Flawil, Switzerland), eluted with aqueous methanol, in a discontinuous gradient: 5% (0-40 min), 5-10% (40-55 min), 10% (55-85 min), 10-15% (85-90 min), 15-25% (90-110 min), 25-50% (110-140 min), 50% (140-160 min), 50-80% (160-180 min), 80-100% (180-200 min) and 100% (200-220 min) at a flow rate of 3 mL/min. The chromatographic profile was registered at 280 and 320 nm by the UV detector C-640 Buchi® (Flawil, Switzerland), and the

data was acquired using the software ECOMAC® 0.238 (Prague, Czech Republic). Two fractions were obtained: F1 (0-120 min), containing phenolic acids and flavonoids and F2 (120-220 min), with flavonoids and tannins. F2 was then sub-fractionated by gel chromatography on a Sephadex® LH-20 (Sigma-Aldrich, Amersham, Sweden) column (35 x 4 cm) using ethanol as mobile phase. All fractionation processes were monitored by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) for polyphenols, providing two major fractions: flavonoids fraction, FF (yield 31.5%) and tannins fraction, TF (yield 14.2%). TF was analyzed by HPLC-DAD-ESI/tandem MS.

2.2 HPLC-DAD-MS

Analyses were carried out in a Hewlett-Packard 1100 chromatograph (Agilent® Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. An Agilent Poroshell 120 EC-C18, 2.7 μm (4.6 x 150 mm) column thermostatted at 35°C was used. The solvents used were: (A) 0.1% formic acid, and (B) acetonitrile. The elution gradient established was from 8% B to 10% B in 5 min, to 25% B in 20 min, to 40% B in 20 min, to 60% B in 5 min and steady at 60% B for more 5 min and re-equilibration of the column using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD at 280, 320 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer, which was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and as turbo gas (400 ºC) for solvent drying (40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupols were set at unit resolution. The ion spray voltage was operated at -4500 V in the negative mode. Method

settings were: declustering potential (DP), -40 V; entrance potential (EP), -10 V; collision energy (CE), -50 V; and cell exit potential (CXP) -3 V. In order to obtain the fragmentation pattern of the parent ion, enhanced product ion (EPI) mode was also applied using the following settings: declustering potential (DP), -50 V; entrance potential (EP), -6 V; collision energy (CE), -25 V; and collision energy spread (CES) 0 V.

2.3 Acid-catalyzed hydrolysis

In order to confirm the nature of the compounds from TF, an acid-catalyzed hydrolysis was performed according to Porter *et al.*(Porter *et al.*, 1986) Briefly, an aliquot of TF dissolved in methanol was mixed with sulphuric acid in buthanol (5%, v/v) and iron-amonium sulfate dodecahydrate in hydrochloric acid 2N (2%, w/v). The mixture was sealed inside a glass vial and heated at 95ºC for 40 min. Then, the product of the hydrolysis was microfiltered and analysed by HPLC-DAD-MS.

2.4 Antioxidant activity

For the antioxidant activity assays, the adequate amount of freeze-dried sample was solubilised in the referred solvent for each test.

2.4.1 DPPH radical assay

Free radical-scavenging activity was evaluated according to the method described by Blois *et al.* (1958). Aliquots of samples (100 μL) were assessed by their reactivity with a methanolic solution of 500 μM DPPH (500 μL) (Sigma-Aldrich, Quimica S.A., Portugal) in the presence of 100 mM acetate buffer, pH 6.0 (1 mL). Reaction mixtures (3 mL) were kept for 30 min at room temperature and in the dark. The decreases in the absorbance were measured at 517 nm. Different dilutions of each of the test compounds were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox® (62.5-1000 μM) (Sigma-Aldrich, Quimica S.A., Portugal). Two independent experiments in triplicate were

performed for each of the assayed compounds. Results were expressed as Trolox® equivalent antioxidant capacity (TEAC), defined as the concentration of the sample solution whose antioxidant capacity is equivalent to a 1.0 mM solution of Trolox® (Antolovich *et al.*, 2002).

2.4.2 ABTS (pH=4) radical assay

The assay was carried out according to Cano (Cano *et al.*, 1998) with minor modifications made by Villaño(Villaño *et al.*, 2004). Free radicals were generated by an enzymatic system consisting of horseradish peroxidase enzyme, its oxidant substrate (hydrogen peroxide) and the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) chromophore. The radical was generated by a reaction between 1.5 mM ABTS (Sigma-Aldrich, Spain), 15 μM hydrogen peroxide (Sigma-Aldrich, Spain) and 0.25 μM peroxidase (Sigma-Aldrich, Spain) in 50 mM glycine-HCl buffer (pH 4.5). The final volume was 60 mL, yielding a final concentration of 30 μM of the $ABTS^{\bullet+}$ radical cation. The blank reference cuvette contained glycine–HCl buffer. Once the radical was formed, the sample was added and the decrease in absorbance was monitored. The assay was carried out at room temperature. The reaction started by adding 100 μL of test sample to 2 mL of $ABTS^{\bullet+}$ solution, the samples were vortexed for 10 s, and the absorbance at 414 nm was measured after 2 min of reaction. Two independent experiments in triplicate were performed for each of the assayed compounds. In each case, six different dilutions were prepared in 50% aqueous methanol and submitted to the reaction. TEAC values were obtained by interpolating the decrease in absorbance on the calibration curve obtained using Trolox® solutions from 62.5 to 500 μM.

2.4.3 ABTS (pH=7) radical assay

In this assay, the $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulphate (2.45 mM, final concentration) (Merck, Darmstadt, Germany) in water. The mixture was allowed to stand in

the dark at room temperature for 12–16 h before use, and then the $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS) at pH 7.4 and equilibrated at 30 ºC to give an absorbance of 0.7±0.02 at 734 nm. Aliquots (50 μL) of 50% aqueous methanol of the test compound were mixed with 2 mL of the $ABTS^{\bullet+}$ preparation, vortexed for 10 s, and the absorbance measured at 734 nm after 4 min of reaction at 30 ºC. Different dilutions of each of the test compounds were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox® (62.5–500 μM). The results were expressed as TEAC values. Two independent experiments in triplicate were performed for each of the assayed compounds.

2.4.4 Ferric reducing power assay

Ferric reducing ability was evaluated according to Benzie and Strain(Benzie and Strain, 1996) with minor modifications. The FRAP reagent contained 10 mM of TPTZ (Sigma-Aldrich, Spain) solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ (Merck, Darmstadt, Germany) and acetate buffer (300 mM, pH 3.6) (1:1:10, v/v/v). Aliquots (100 μL) of 50% aqueous methanol of the test compounds were added to 3 mL of the FRAP reagent, and the absorbance was measured at 593 nm after incubation at room temperature for 6 min, using the FRAP reagent as blank. Different dilutions of each of the test compounds were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox® (31.25-1000 μM). The results were expressed as TEAC values. Two independent experiments in triplicate were performed for each of the assayed compounds.

3.Results and Discussion

3.1 HPLC-DAD-MS analyses

The HPLC-DAD chromatogram of the tannin-rich fraction obtained from an oil-free infusion of *C. citratus* (TF) is shown in **Figure II.9**. The

tentative identities, retention times, UV maxima, and recorded molecular and MS^2 fragment ions for individual components are presented in **Table II.5**. A total of 15 compounds were tentatively identified including two distinct types of flavonoids: flavan-3-ols and flavan-flavanone hetero-dimers, these latter being the most abundant ones.

Compounds **1-4** showed a UV spectral shape characteristic of flavan-3-ols with a single maximum at 278 nm. Compound **2** ($[M-H]^-$ at *m/z* 289) was identified as catechin as confirmed by comparison with a commercial standard. Compounds **1**, **3** and **4** were identified as dimeric procyanidins based on their molecular ion ($[M-H]^-$ at *m/z* 577) and MS^2 fragmentation pattern showing the typical *retro* Diels-Alder fission (RDA; -152 amu, *m/z* at 425), RDA + water loss (-170 amu, *m/z* at 407), heterocyclic ring fission (-126 amu, *m/z* at 451), and cleavage of the interflavanic bond following the quinone-methide mechanism (QM; -288 amu, *m/z* at 289). By comparison with the relative retention times of procyanidins previously identified in the laboratory, it was possible to identify compounds **1**, **3** and **4** as the procyanidin dimers B_3, B_4 and B_2, respectively.

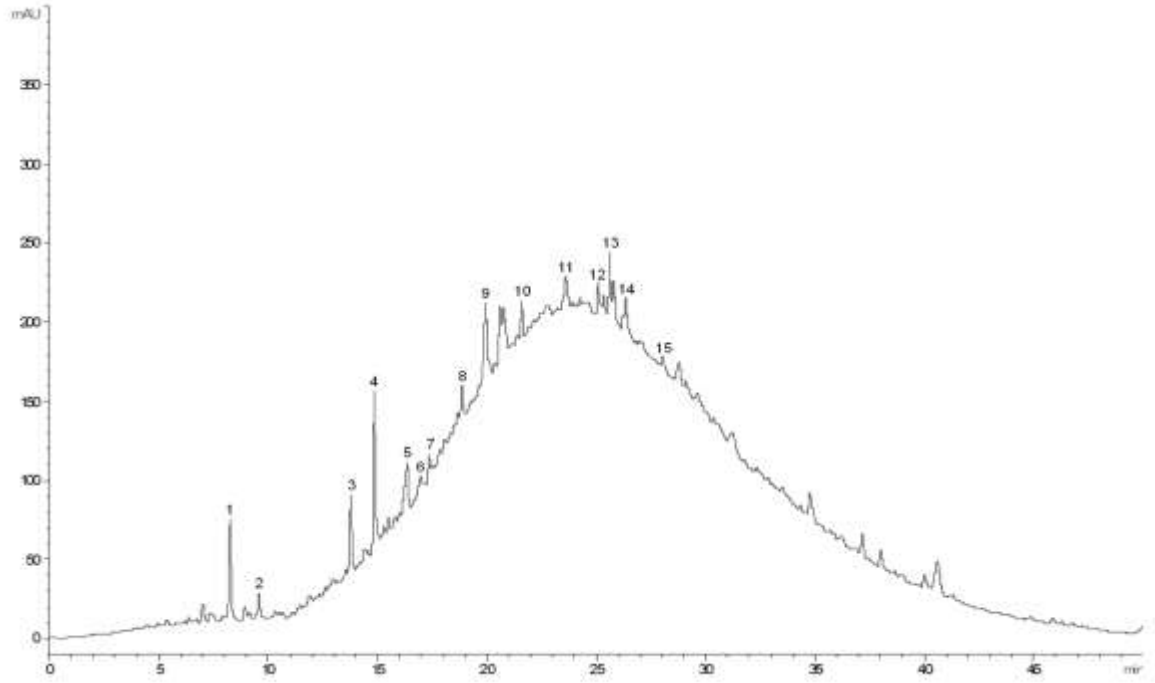


Figure II.9. HPLC-DAD profile of tannin-rich fraction from *Cymbopogon citratus* leaves, registered at 280 nm.

Compounds **5-15** exhibited a different profile of UV spectrum, showing maximum absorbance at 278-280 nm but also an inflexion between 331 and 349 nm, and their mass spectra suggest that they could correspond to flavan-flavanone hetero-dimers similar to those reported in sorghum (Gujer *et al.*, 1986; Krueger *et al.*, 2003).

The detected compounds belonged to a series of dimers differing in masses of ±16 amu and ±162 amu depending on the number of hydroxy groups on ring-B and/or hexosyl residues, respectively, and their identities were assigned based on their molecular ions and MS^2 fragmentation patterns. It has been indicated that the position of elementary units in proanthocyanidin oligomers could be deduced through the analysis of the product ions derived from the quinone methide (QM) cleavage of the interflavan bond, where lower (terminal) units would be released as such, while the upper (extension) units suffer a structural rearrangement yielding ions 2 Da lower than the original flavan constituents (Friedrich and Eberhardt, 2000; Gu *et al.*, 2003). Thus, for instance, terminal eriodyctiol (MW 288) and narigenin (MW 272) would be expected to produce negative ions at *m/z* 287 and 271, respectively, whilst extension luteoliflavan and apigeniflavan units could be expected to produce them at *m/z* 272 and 256, respectively.

Tentative identities for compounds **5-15** were assigned based on those assumptions and are indicated in **Table II.5** and their structures depicted in **Figure II.10**.

Compound **12** presented a molecular ion $[M-H]^-$ at *m/z* 543 releasing MS^2 fragment ions at *m/z* 423 (-120 amu) and 407 (-136 amu) from the RDA fission of mono- and di-hydroxylated B-ring units, respectively; the higher abundance of the ion at *m/z* 407 suggested that it was produced from the cleavage of the upper (extension) unit of a di-hydroxylated B-ring, which was, therefore, associated to a luteoliflavan. The other two product ions at *m/z* 272 and 271 would derive from the interflavan cleavage and could be

attributed to the upper luteoliflavan unit and a terminal naringenin, respectively. Thus, the compound was tentatively identified as the dimer luteoliflavan-naringenin. Similar reasoning was applied to assign compounds **14** and **15** as the dimers apigeniflavan-naringenin and luteoliflavan-eriodictyol, respectively. The other hetero-dimers contained one or two hexosyl substituents linked to the aglycone(s) through an oxygen bridge, as deduced from the observation of losses of 162 amu in their MS^2 fragmentation spectra. Compound **9** ($[M-H]^-$ at *m/z* 705) showed a majority product ion at *m/z* 543 (-162 amu, loss of a hexosyl residue); the ion at *m/z* 287 would be due to further QM cleavage of the previous ion, suggesting that eriodictyol hexoside would be the terminal unit.

The other hetero-dimers contained one or two hexosyl substituents linked to the aglycone(s) through an oxygen bridge, as deduced from the observation of losses of 162 amu in their MS^2 fragmentation spectra. Compound **9** ($[M-H]^-$ at *m/z* 705) showed a majority product ion at *m/z* 543 (-162 amu, loss of a hexosyl residue); the ion at *m/z* 287 would be due to further QM cleavage of the previous ion, suggesting that eriodictyol hexoside would be the terminal unit. The other two fragment at *m/z* 585 (-120 amu) and 569 (-136 amu) could be attributed to RDA fissions of the upper and lower units, respectively, taking into account relative abundances, which suggested an apigeniflavan nature for the extension unit. Therefore, the compound was tentatively assigned as apigeniflavan-eriodictyol-*O*-hexoside. Based on similar considerations, compounds **10**, **11** and **13** were respectively identified as luteoliflavan-eriodictyol-*O*-hexoside, luteoliflavan-naringenin-*O*-hexoside and apigeniflavan-naringenin-*O*-hexoside.

Compound **8** ($[M-H]^-$ at *m/z* 851) showed major fragment ions at *m/z* 689 and 527 from the consecutive loss of two hexosyl residues, whereas another ion at *m/z* 731 (-120 amu) pointed to the existence of an upper apigeniflavan unit, so it could be tentatively identified as apigeniflavan-*O*-hexosyl-naringenin-*O*-hexoside. Similarly, according to its molecular ion, compound **5** was assigned as luteoliflavan-*O*-hexosyl-eriodictyol-*O*-hexoside.

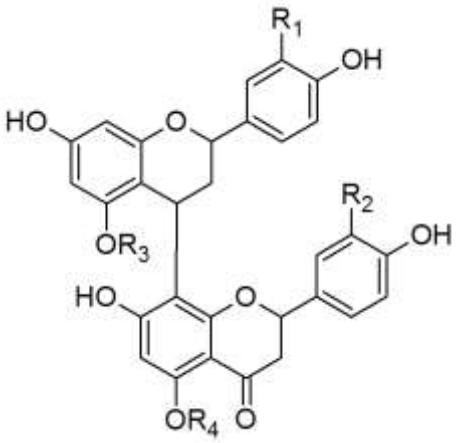
Compounds **6** and **7** presented the same molecular ion at *m/z* 867 and similar fragmentation pattern with two main fragments ions at *m/z* 705 and 543 from the consecutive loss of two hexosyl residues, and other two at *m/z* 747 (-120 amu) and 731 (-136 amu) attributable to RDA cleavages.

These compounds were tentatively assigned based on the relative abundances of these latter ions.

Table II.5. Phenolic characterization of tannin-rich fraction from *Cymbopogon citratus* infusion, by HPLC-DAD-tandem MS.

Peak	Tentative Identification	Rt (min)	λmaxima (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)
1	Procyanidin B3*	8.25	278	577	451(13), 425(46), 407(100), 289(54)
2	Catechin*	9.59	278	289	245(100), 221(28), 203(50), 163(45), 137(22), 125(66)
3	Procyanidin B4*	13.78	278	577	451(30), 425(65), 407(91), 289(100)
4	Procyanidin B2*	14.85	278	577	451(33), 425(72), 407(94), 289(100)
5	Luteoliflavan-*O*-hexosyl-eriodictyol-*O*-hexoside	16.34	280, 338	883	747(11), 721(100), 559(77)
6	Apigeniflavan-*O*-hexosyl-eriodictyol-*O*-hexoside	17.34	280, 338	867	747(5), 731(2), 705(100), 543(86)
7	Luteoliflavan-*O*-hexosyl-naringenin-*O*-hexoside	18.85	280, 342	867	747(2), 731(3), 705(100), 543(74)
8	Apigeniflavan-*O*-hexosyl-naringenin-*O*-hexoside	19.95	280, 339	851	731(3), 689(100), 527(80)
9	Apigeniflavan-eriodictyol-*O*-hexoside	20.61	280, 331	705	585(16), 569(5), 543(100), 287(2)
10	Luteoliflavan-eriodictyol-*O*-hexoside	21.58	280, 349	721	585(13), 559(100), 287(3)
11	Luteoliflavan-naringenin-*O*-hexoside	23.60	279, 344	705	585(2), 569(4), 543(100), 271(2)
12	Luteoliflavan-naringenin	25.09	280, 342	543	423(31), 407(100), 272(3), 271(15)
13	Apigeniflavan-naringenin-*O*-hexoside	25.71	278, 335sh	689	569(5), 527(100), 271(2)
14	Apigeniflavan-naringenin	26.30	279, 336	527	407(100), 271(11), 256(2)
15	Luteoliflavan-eriodictyol	28.77	278, 341	559	423(100), 287(13), 272(3)

sh: shoulder; *confirmed by a commercial standard



Compound	R_1	R_2	R_3	R_4
5	OH	OH	hex	hex
6	H	OH	hex	hex
7	OH	H	hex	hex
8	H	H	hex	hex
9	H	OH	H	hex
10	OH	OH	H	hex
11	OH	H	H	hex
12	OH	H	H	H
13	H	H	H	hex
14	H	H	H	H
15	OH	OH	H	H

hex: hexosyl residue

Figure II.10. Proposed structures for compounds **5-15** identified in tannin-rich fraction from *Cymbopogon citratus* leaves.

Thus, the ion at *m/z* 747 was more abundant in compound **6** which might suggest an upper apigeniflavan unit, so that it could be identified as apigeniflavan-*O*-hexosyl-eriodictyol-*O*-hexoside. In contrast the ion at *m/z* 731 was more abundant in compound **7** pointing to an upper luteoliflavan unit, allowing its tentative identification as luteoliflavan-*O*-hexosyl-naringenin-*O*-hexoside. The nature and substitution position of the hexosyl residues on the different aglycones cannot be established from the available data, although they might be speculated to be glucose probably linked to the hydroxyl group at C_5 of the corresponding flavan unit, according to the previous identifications made by Gujer (Gujer *et al.*, 1986) and Krueger(Krueger *et al.*, 2003) in sorghum.

3.2 Acid-catalyzed hydrolysis

The product from TF acid hydrolysis was characterized by HPLC-DAD-MS and apigenidin and luteolinidin molecules were detected (data not shown). These results confirm the presence of apigeniflavan and luteoliflavan residues in the structure of the tannin oligomers present in TF, respectively.

3.3 Antioxidant activity

Table II.6 shows the TEAC values for the lipid-free infusion (CcI) and tannin fraction (TF) of *C. citratus* obtained by different assays. In all assays, TF exhibited higher antioxidant capacity (lower TEAC values). In the DPPH assay, the TEAC value for TF was 0.61, 2.33 times more potent than CcI.

Table II.6. Antioxidant activity of CcI and TF from *Cymbopogon citratus*.

Sample	TEAC*			
	DPPH•	ABTS• (pH=4)	ABTS• (pH=7)	Fe^{3+}
CcI	1.40 ± 0.06	2.29 ± 0.02	0.83 ± 0.04	2.63 ± 0.10
TF	0.61 ± 0.07	1.59 ± 0.05	0.65 ± 0.06	0.38 ± 0.07

*TEAC (Trolox®-Equivalent Antioxidant Capacity): Amount of the samples (mg/mL) that has the same anti-radical activity of Trolox® 1 mM. The results are expressed as mean±SD of three independent experiments.

Nevertheless, the TEAC value of CcI, which corresponds to an EC_{50} of 28.99μg/mL, is substantially better than another lemongrass infusion previously mentioned in the literature, which presents an EC_{50} of 41.72μg/mL(Tavares *et al.*, 2015). In the ABTS (pH=4) method, TF and CcI showed TEAC values of 1.59 and 2.29, respectively; whereas in the ABTS (pH=7) assay, the samples gave TEAC values of 0.65 and 0.83, respectively. In both cases, TF was about 1.3 times more efficient in neutralizing the ABTS• radical than CcI. However, the two samples happened to be significantly more potent at pH=7 than in an acidic environment, which might be important when considering physiological conditions. In the FRAP assay, TF (TEAC value of 0.38) was almost 7 times more efficient than CcI (2.63). In the case of CcI, the antioxidant capacity increases as follows: Fe^{3+} < ABTS (pH=4) < DPPH < ABTS (pH=7), while TF antioxidant power increases: ABTS (pH=4) < ABTS (pH=7) < DPPH < Fe^{3+}. This may be explained by the fractionation process, leading to a different matrix and composition with a distinct chemical behaviour against the tested oxidant species. Nevertheless, the tannin fraction proved to be fairly more active in inactivating the potentially harmful oxidant entities

than the crude extract obtained by infusion. This data seems to be supported by previous studies in *Sorghum bicolor*, which showed that this type of condensed tannins has a very important contribution to the antioxidant activity of the whole plant (Bors *et al.*, 2000; Choi *et al.*, 2007).

4. Conclusions

The present work allowed the identification of hetero-dimeric flavan structures for the first time in *Cymbopogon citratus*. These flavonoid oligomers consist of apigeniflavan or luteoliflavan units linked to a flavanone, either naringenin or eriodictyol, and occur as aglycone and glycosylated forms. The detected proanthocyanidin hetero-dimers, along with some common procyanidin dimers, constitute the main compounds in the tannin fraction of *C. citratus* infusion. The findings of this study support the antioxidant potential of this plant and emphasize the contribution of the tannin fraction to this activity.

D. Gastroprotective effect of *Cymbopogon citratus* infusion on acute ethanol-induced gastric lesions in rats

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Abstract

Ethnopharmacological relevance: Treatment of gastric ulcers with medicinal plants is quite common in traditional medicine worldwide. *Cymbopogon citratus* (DC.) Stapf leaves infusion has been used in folk medicine of many tropical and subtropical regions to treat gastric disturbances.

Aim of the study: The aim of this study was to assess the potential gastroprotective activity of an essential oil-free infusion from *C. citratus* leaves in acute gastric lesions induced by ethanol in rat.

Materials and Methods: The study was performed on adult male Wistar rats (234.0 ± 22.7 g) fasted for 24 hours but with free access to water. The extract was given orally before (prevention) or after (treatment) intragastric administration of absolute ethanol. Effects of dose (28 or 56 mg/kg of body weight) and time of contact of the extract with gastric mucosa (1 or 2 hours) were also assessed. Animals were sacrificed, being the stomachs removed and the lesions were assessed by macroscopic observation and histopathology.

Results: *C. citratus* extract, given orally before or after ethanol, significantly ($P<0.01$) reduced gastric mucosal injury compared with control group (vehicle + ethanol). The effect does not appear to be dose-dependent. Results also suggested that the extract is more effective when the time of contact with gastric mucosa increases.

Conclusions: The results of this assay confirm the gastroprotective activity of *C. citratus* extract on experimental gastric lesions induced by ethanol, contributing for the pharmacological validation of its traditional use.

Keywords: *Cymbopogon citratus*; lemongrass; infusion; gastric ulcer; gastroprotective.

1. Introduction

Peptic ulcer disease is a serious gastrointestinal disease that affects a considerable number of people in the world and has a significant impact on the quality of life (Yuan *et al.*, 2006). Many drugs currently available for the treatment of peptic ulcers have decreased the morbidity rates, but limitations still exist, such as incidence of relapses and adverse effects (Borrelli and Izzo, 2000; Schmeda-Hirschmann and Yesilada, 2005). *Cymbopogon citratus* (DC.) Stapf (Poaceae), also known as lemongrass, is a perennial herb native to India and widely cultivated in other tropical and subtropical regions. Infusions and decoctions of *C. citratus* leaves have been used in popular medicine to treat a wide variety of health problems such as feverish conditions, nervous and gastrointestinal disorders (Carlini *et al.*, 1986). It is also recommended in folk medicine to treat gastric disturbances, stomachache, gastritis and ulcers (de Albuquerque *et al.*, 2007; Novais *et al.*, 2004; Tene *et al.*, 2007). Studies on *C. citratus* leaves infusions and decoctions have shown antioxidant and anti-inflammatory activities (Cápiro *et al.*, 2001; Carbajal *et al.*, 1989; Cheel *et al.*, 2005; Figueirinha *et al.*, 2010, 2008; Francisco *et al.*, 2013, 2011; Pereira *et al.*, 2009) and revealed the presence of several polyphenols, including tannins, phenolic acids and flavonoids (Cheel *et al.*, 2005; Figueirinha *et al.*, 2008; Francisco *et al.*, 2014; Marques and Farah, 2009; Pereira *et al.*, 2009). Those properties may be indicative of the potential benefit in the gastrointestinal tract, mainly on gastric mucosal injury generated by oxidative damage. However, to our knowledge there are no pharmacological studies regarding the potential gastroprotective activity of this medicinal plant in literature.

This study was therefore carried out in order to assess the gastroprotective activity of an essential oil-free infusion from *C. citratus* dry leaves in acute gastric lesions experimentally induced by ethanol in rats.

2. Materials and methods

2.1 Plant material and extract preparation

Dry leaves of *Cymbopogon citratus* (DC.) Stapf were acquired from Ervital® (Mezio, Castro Daire, Portugal). The plant was cultivated in the region of Mezio, Castro Daire (Portugal), in a greenhouse located 1000 m above sea level. The identity of the plant was confirmed by Prof. Dr. J. Paiva (Department of Botany, University of Coimbra, Portugal). A voucher specimen is deposited in the herbarium of the Faculty of Pharmacy, University of Coimbra (A. Figueirinha 0109).

The extract was prepared as previously described by Figueirinha and co-workers (Figueirinha *et al.*, 2008). Briefly, an infusion was obtained by adding 150 mL of boiling water to 5 g of the powdered plant material and left to stand at room temperature for 15 minutes. The infusion was filtered under vacuum and the volume was made up to 150 mL with the same solvent. Afterwards, an essential oil-free infusion was obtained by repeatedly washing the infusion with *n*-hexane to remove the less polar compounds; the aqueous phase was concentrated in a rotavapor and then freeze-dried (extraction yield: 14%). Before the experiment, the extract was solubilized in water (vehicle) and given to rats, by gavage, at a dose of 28 mg/kg of body weight (d1) or 56 mg/kg of body weight (d2). These doses were calculated by extrapolation from the human dose recommended in traditional medicine (2 g of dried leaf/150 mL of water) (Carlini *et al.*, 1986).

2.2 Animals

Adult male Wistar rats (234.0±22.7 g) were purchased from Charles River Laboratories (Barcelona, Spain). The rats were housed at least for 7 days before experiments in the local bioterium under standard laboratory conditions, which include a temperature of 22±1ºC, relative humidity of about 50-60% and a controlled 12-h light cycle beginning early in the morning. Rats were deprived of food for 24 hours prior to experiments, but allowed free access to water. During the period of starvation animals were housed in wire-bottom cages to prevent coprophagy. Animal experimentation in this study was conducted in accordance

with the European guidelines for laboratory animal use and care (86/609/EEC) and the project was approved by the Portuguese Veterinary General Division (Reference no. 99 – 0420/000/000, 9/11/2009).

2.3 Ethanol-induced gastric lesions assay

Rats were divided into nine experimental groups (n = 6-9). Absolute ethanol administered orally by gavage at a single dose of 1 mL/rat was used as ulcerogenic agent. Group I received vehicle (water) orally one hour before ethanol (positive control). The animals were sacrificed one hour after ulcer induction. Animals from group II received orally only vehicle, and one hour later they were sacrificed (negative control). Animals from group III received orally only extract, and one hour later they were sacrificed (negative control). Animals from groups IV, V and VI were given extract orally prior to ulcer induction (prevention groups). Animals from group IV received dose d1 of the extract one hour before ethanol administration. Animals from group V received dose d2 of the extract one hour before ethanol administration. Animals from group VI received dose d1 of the extract two hours before ethanol administration. One hour after ulcer induction the animals from groups IV-VI were sacrificed. Animals from groups VII, VIII and IX received extract orally after ulcer induction by ethanol (treatment groups). One hour after ethanol administration, the animals received a dose d1 (groups VII and IX) or d2 (group VIII) of the extract. They were sacrificed one hour (groups VII and VIII) or two hours (group IX) after extract administration. After the animals were sacrificed, each stomach was removed, opened along the greater curvature, rinsed with physiological saline and photographed. The gastric mucosa was carefully examined, macroscopically and by histopathology, and scored.

2.4 Macroscopic assessment of gastric mucosal lesions

Gastric mucosa was examined macroscopically and each lesion was graded and scored based on its incidence and severity according to an arbitrary grading system. The grade of severity was attributed differently in each parameter: loss of mucosal folds (0, 1, 2, 3); haemorrhagic areas (0, 1, 2, 3); necrotic areas/ulcers (0,

2, 4, 6); perforation (0, 10). The ulcer index (U.I.) for each stomach was the sum of scores of all lesions. The U.I. for each experimental group was reported as median (minimum – maximum). The significance of differences between groups was assessed by Kruskal-Wallis test, followed by Mann-Whitney U test, when appropriate. $P<0.05$ *versus* control was taken as significant.

2.5 Microscopic assessment of gastric mucosal lesions

After macroscopic examination of the stomach, gastric tissue samples from the lesion sites were prepared for histopathological studies. The fragments were fixed in 4% neutral phosphate-buffered formalin, embedded in paraffin blocks, cut in 3 μm thick sections and stained with hematoxylin and eosin. The slides were examined under microscope and assessed for histopathological changes such as congestion, oedema, haemorrhage and necrosis.

3. Results

The severity of gastric mucosal damage in the experimental groups, as measured by U.I., is listed in **Table II.7**. Rats treated only with vehicle and *C. citratus* extract (groups II and III) showed a normal morphology of the gastric mucosa. In the positive control group, treated orally with vehicle and absolute ethanol, severe and widespread damage of the glandular mucosa was observed macroscopically and the U.I. was found to be very high (median: 9.0). Ethanol administration produced the expected characteristic haemorrhagic and necrotic mucosal lesions, consisting of elongated bands usually parallel to the long axis of the stomach, submucosal oedema and loss of epithelial cells (**Figure II.11a**). In accordance with the macroscopic assessment, the histological examination of the gastric tissue samples revealed haemorrhagic areas, mucosal atrophy and presence of inflammatory infiltrate (**Figure II.11b**).

In the prevention groups, treated orally with *C. citratus* extract prior to ethanol, an important reduction in both number and severity of gastric lesions was observed (**Figure II.12**). The U.I. significantly decreased in all prevention groups ($P<0.01$) compared to the positive control group. Similarly, when administered

after ethanol administration (treatment groups), *C. citratus* extract also produced a significant reduction in the U.I. ($P<0.01$) compared to the positive control group. However, the protective effect of the extract does not seem to be dose-dependent, as no significant differences in the U.I. were found across the doses used, both in prevention (group IV *versus* V; $P=0.143$) and treatment groups (group VII *versus* VIII; $P=0.154$). Although not statistically significant, the gastroprotective effects of the extract were more pronounced when time of contact with gastric mucosa increased to 2 hours (group VI and IX) compared to 1 hour (groups IV and VII).

Table II.7. Effect of essential oil-free infusion from *Cymbopogon citratus* leaves on gastric lesions induced by ethanol in rats.

Experimental group	Administered product(s)	Dose (mg/kg)	Time of contact (h)	Ulcer Index (U.I.) [median (min-max)]
Control groups:				
Group I	Vehicle, ethanol	-	-	9.0 (5 – 12)
Group II	Vehicle	-	-	0.0
Group III	Extract	28	1	0.0
Prevention groups:				
Group IV	Extract, ethanol	28	1	3.5 (2 – 4)*
Group V	Extract, ethanol	56	1	4.0 (3 – 5)*
Group VI	Extract, ethanol	28	2	2.5 (1 – 4)*
Treatment groups:				
Group VII	Ethanol, extract	28	1	4.0 (3 – 5)*
Group VIII	Ethanol, extract	56	1	4.0 (3 – 6)*
Group IX	Ethanol, extract	28	2	3.0 (1 – 4)*

* $P<0.01$, compared to positive control (group I) (Kruskal-Wallis test, followed by Mann-Whitney U test).

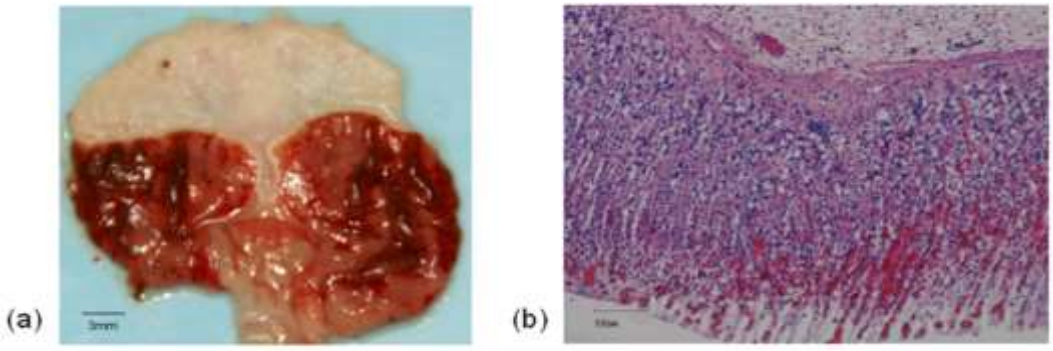

Figure II.11. Ethanol-induced gastric injury in rats. (**a**) Representative macroscopic aspect of the stomach, showing severe and widespread damage of the glandular mucosa one hour after ethanol administration. (**b**) Histological section of gastric mucosa, showing haemorrhagic areas, mucosal atrophy and presence of inflammatory infiltrate (HE staining).

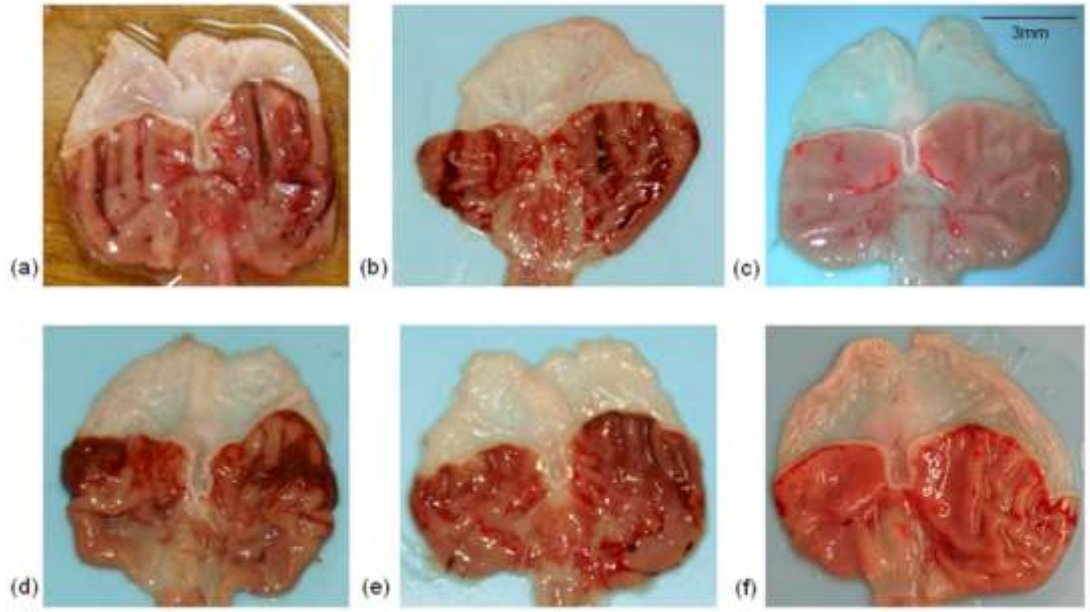

Figure II.12. Representative macroscopic aspect of the effects of *Cymbopogon citratus* extract on ethanol-induced gastric lesions in rats. (a) Stomach treated with extract, dose 28 mg/kg, one hour before ethanol (group IV). (b) Stomach treated with extract, dose 56 mg/kg, one hour before ethanol (group V). (c) Stomach treated with extract, dose 28 mg/kg, two hours before ethanol (group VI). (d) Stomach treated for one hour with extract, dose 28 mg/kg, after ethanol administration (group VII). (e) Stomach treated for one hour with extract, dose 56 mg/kg, after ethanol administration (group VIII). (f) Stomach treated for two hours with extract, dose 28 mg/kg, after ethanol administration (group IX).

4. Discussion

The formation of gastric mucosal lesions following ethanol administration seems to occur both directly and indirectly through several mechanisms. Gastric mucosal damage induced by ethanol has been reported to involve a depletion of gastric defensive mechanisms, including disruption of the mucosal barrier, gastric mucus depletion, reduced gastric blood flow and alterations in permeability, that contribute to the development of the haemorrhagic and necrotic aspects of tissue injury (Glavin and Szabo, 1992). Furthermore, some studies suggest that oxygen-derived reactive species and lipid peroxidation are associated with gastric damage induced by ethanol. This data suggest that antioxidant compounds could be active in this experimental model, producing gastroprotective effects (Glavin and Szabo, 1992; La Casa *et al.*, 2000).

Essential-oil free infusion from *Cymbopogon citratus* leaves, administered before (prevention) or after (treatment) ethanol, reduced significantly the incidence and severity of gastric lesions and consequently the U.I.. These results indicate that *C. citratus* extract, at doses equivalent to the human dose recommended in traditional medicine, display a gastroprotective effect against ethanol-induced ulcer.

A previous phytochemical study of the essential oil-free infusion from *C. citratus* used in this study has indicated the presence of tannins, phenolic acids (caffeic and *p*-coumaric acid derivatives) and flavonoids (Figueirinha *et al.*, 2008). In the flavonoid fraction, representing 6.1% of the extract, several *O*- and *C*-glycosylflavones of apigenin and luteolin were identified (Francisco *et al.*, 2014). Many compounds from these chemical classes have been shown to possess anti-ulcer properties (Borrelli and Izzo, 2000). Tannins have radical-scavenger properties and, at low concentrations, are known to create a layer in the mucosa and to increase the resistance to chemical and mechanical injury or irritation (Borrelli and Izzo, 2000). Recently, a different group of flavanic dimmers have been identified in lemongrass infusion, which have been correlated with a pronounced anti-radical activity (Costa *et al.*, 2015a). Phenolic acids, such as caffeic, *p*-coumaric, ferulic and hydroxycinnamic acids, display anti-ulcer activity in several acute gastric ulcer models (Barros *et al.*, 2008). It is well known that many flavonoids have antisecretory and cytoprotective properties in different experimental models of gastric ulcer (La Casa *et al.*, 2000; Mota *et al.*, 2009). In particular, several plants with anti-ulcer activity which contain luteolin and luteolin *O*- and *C*-glycosides have been reported (Batista *et al.*, 2004; Coelho *et al.*, 2009, 2006; Min *et al.*, 2006; Yesilada *et al.*, 2000). Therefore, the polyphenolic chemical composition of the extract may contribute, at least in part, to the gastroprotective effects observed in this study.

Literature reports that many polyphenols have antiulcer activity probably because of their antioxidant properties, which could prevent the formation of free radicals in the body and also minimize injuries by oxidative reactions (La Casa *et al.*, 2000; Repetto *et al.*, 2002). In previous studies our group demonstrated the *in*

vitro antioxidant and anti-inflammatory activity of the essential oil-free infusion from *C. citratus* used in this study and of different fractions obtained from it (Figueirinha *et al.*, 2010, 2008), providing a possible mechanism to explain the protection produced by the plant extract against ethanol-induced gastric lesions.

5. Conclusions

In conclusion, the results of this study suggest a gastroprotective activity of *C. citratus* extract against ethanol-induced gastric lesions, contributing to the pharmacological validation of its traditional use. In the future, further pharmacological evaluations are required to identify and isolate the active gastroprotective compounds in the plant as well as elucidating their mechanisms of action.

E. *In vivo* evaluation of anti-inflammatory and analgesic activities of flavonoid and tannin fractions from *Cymbopogon citratus* infusion

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Evaluation of anti-inflammatory and analgesic activities of *Cymbopogon citratus in vivo* – polyphenols contribution.

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Abstract

Cymbopogon citratus is one of the most common herbs used in folk medicine due to its anti-inflammatory and antioxidant properties. Taking into account these properties showed on *in vitro* assays, the aim of this work was to evaluate the anti-inflammatory and analgesic activities of *C. citratus* leaves infusion (CcE), and its flavonoid-rich (CcF) and tannin-rich (CcT) fractions. The evaluation of the anti-inflammatory activity was performed in the carrageenan-induced rat paw oedema model. Both central and peripheral analgesic activities were evaluated in mice through the hot plate test and the acetic acid-induced writhing test, respectively.

In the acute inflammation model, the statistically significant results obtained in percentage of oedema inhibition were 70.80% and 82.30% for CcE (34.12 mg/kg and 68.24 mg/kg, respectively), 59.00% for CcF (7.42 mg/kg), 61.00% for CcT (5.96 mg/kg) and 84.00% for positive control group (10 mg/kg). For the peripheral pain evaluation, statistically significant results showed a pain reduction of 57.00% for CcE (136.48 mg/kg), 54.60% for CcF (14.8 mg/kg), 52.20% for CcT (11.92 mg/kg) and 83.00% for positive control group.

This study demonstrates that *C. citratus* infusion compounds are able to reduce inflammation and peripheral pain *in vivo*, with polyphenols showing a significant contribution for these activities.

Keywords: *Cymbopogon citratus*, lemongrass, *in vivo,* inflammation, pain, phenolic compounds.

1. Introduction

C*ymbopogon citratus* (DC.) Stapf, commonly known as lemongrass, is an Indian native herb belonging to the Poaceae, which comprises approximately 500 genus and 8000 herb species (Barbosa *et al.*, 2008). *C. citratus* is used in folk medicine with a wide range of indications, such as digestive and nervous disorders, inflammation, pain, fever and diabetes (Lorenzetti *et al.*, 1991). It is also consumed as an aromatic drink and is widely used in traditional cuisine due to its lemon flavor (Figueirinha *et al.*, 2008). Moreover, *C. citratus* leaves are a source of essential oil used both in the flavor and fragrance industries and for the production of insect repellents and disinfectants (Negrelle and Gomes, 2007; Rauber *et al.*, 2005).

Because of its economic impact, most phytochemical studies are centered in the volatile compounds of *C. citratus* leaves and only essential oil chemical composition is well known (Kasali *et al.*, 2001). However, many of the suggested therapeutic activities of lemongrass have been attributed to the non-volatile phenolic compounds present in leaves, which have gained attention especially by their antioxidant and radical-scavenging activities. This fact has increased both industrial and academic interest on the non-volatile composition of the plant (Arce *et al.*, 2004; Figueirinha *et al.*, 2008). Presently, it has been proven the presence of a great variety of constituents in *C. citratus* leaves, such as phenolic acids, alkaloids, alcohols and, more important, flavonoids and tannins, which may be responsible for some therapeutic activities such as anti-inflammatory activity (Figueirinha *et al.*, 2010; Negrelle and Gomes, 2007; Vendruscolo *et al.*, 2005).

Considering that inflammation may lead to various diseases – such as rheumatoid arthritis, inflammatory bowel disease or psoriasis – and current steroidal and non-steroidal anti-inflammatory drugs used to treat inflammatory disorders can develop many adverse effects, the discovery of new and safer anti-inflammatory agents continue to be an issue of high interest. In this field, plants used in folk medicine become excellent research candidates for their potential content in compounds to lead to effective herbal formulations from standardized active extracts.

To our knowledge, there are no pharmacological studies that evaluate the anti-inflammatory potential of *C. citratus* leaves infusion and its phenolic compounds on *in vivo* models, even if in a previous study of our group it has been reported that, under inflammatory conditions, an essential oil-free infusion of *C. citratus* leaves allowed cell viability and conferred significant reduction of nitric oxide production in dendritic cells stimulated with lipopolysaccharide (Figueirinha *et al.*, 2010). More recently, our group found more evidence of the anti-inflammatory mechanisms behind the beneficial effects of this plant, and mainly due to its phenolic compounds in vitro (Francisco *et al.*, 2014, 2013, 2011).

Based on these findings, the aim of the present work was to evaluate the anti-inflammatory and analgesic activities of an essential oil-free infusion (CcE), a flavonoid-rich fraction (CcF) and a tannin-rich fraction (CcT) of *C. citratus* leaves on animal models of acute inflammation and pain.

2. Materials and methods

2.1. Plant material and infusion preparation

Dry leaves of *C. citratus* (DC.) Stapf were purchased from ERVITAL® (Mezio, Castro D'Aire, Portugal). The plant was cultivated in the region of Mezio, Castro d'Aire (Portugal), in a greenhouse at 1000 m above sea level and was collected in September 2007. 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 plant identification was confirmed by Dr. J. Paiva (Department of Life Sciences, University of Coimbra, Portugal).

CcE was obtained as previously described by Figueirinha *et al.* (Figueirinha *et al.*, 2008). Briefly, the infusion was prepared by adding boiling water to the plant material (30:1 v/w) and left for 15 min. After the extraction, the mixture was filtered under vacuum and it was washed three times with *n*-hexane to remove the less polar compounds. The aqueous phase was concentrated using a rotary evaporator to a small volume, freeze-dried, and kept at -20ºC, in N_2 atmosphere, until use. All subsequent study was performed with this essential oil-free infusion (CcE). For *in vivo* experiments the infusion was dissolved in distilled water.

Administrated doses were calculated based on the traditionally used doses, taking into consideration the yield of the extraction process and the conversion into animal doses, according to the FDA (Food and Drug Administration) guidelines. In order to examine a possible dose-dependent effect, two different doses were used: D'1 and D'2 (dose and double dose for the anti-inflammatory assay), and D''1 and D''2 (dose and double dose for the analgesic assay), according to the animal model used.

2.2. Infusion fractionation

The flavonoid and tannin-rich fractions were obtained using a reverse phase semipreparative column Lichroprep® RP-18 (310 mm x 25 mm, particle sizes 40-63 μm), Merck (Darmstadt, Germany), eluted with water and aqueous methanol solutions. Tannin-rich fraction (CcT), phenolic acids fraction and a mixture of phenolic acids and flavonoids were obtained. Dry residue of that mixture 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, as previously described (Figueirinha *et al.*, 2010). A flavonoid-rich fraction (CcF) was obtained. All the fractioning processes described above were monitored by HPLC and thin-layer chromatography.

The tannin and flavonoid-rich fractions were concentrated under reduced pressure at 40°C and freeze-dried. Again, before the *in vivo* experiments, the fractions were dissolved in distilled water and the doses administrated were calculated taking into consideration the yield of the fractioning process, according to Figueirinha *et al.* (Figueirinha *et al.*, 2010), and converted into animal doses.

2.3. Reagents and drugs

n-Hexane was purchased from Carlo Erba Reagenti SpA (Milan, Italy). Acetic acid was purchased from J.T. Baker (Deventer, Holand). Ketamine (Ketalar, 7.7 mg/kg) was purchased from Parke-Davis, Pfizer (Seixal, Portugal) Chlorpromazine (Largatil, 2.3 mg/kg) was purchased from Rhône-Poulenc Rores, Laboratório Vitória SA (Amadora, Portugal). Diclofenac sodium (Voltaren 75

mg/3mL) was purchased from Novartis Pharmaceuticals SA (Barcelona, Spain). Morphine hydrochloride and carrageenan-κ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were purchased from Merck (Darmstadt, Germany).

2.4. Animals

Male Wistar rats weighing 120-220 g and male mice weighing 20-30 g were obtained from Charles River (Barcelona, Spain). The animals were maintained with food and water *ad libitum* and kept at 22 ± 1ºC with controlled 12-hour light/dark cycle at the Faculty of Pharmacy (University of Coimbra). The animals were allowed to adapt to the laboratory for 7 days before testing.

For evaluation of *C. citratus* anti-inflammatory activity, male Wistar rats were fasted with free access to water at least 24 h prior to experiments, and to study the analgesic activities, male mice were fasted with free access to water at least 18 h prior to experiments. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Directive 2010/63/EU.

2.5. Pharmacological assays

2.5.1. The carrageenan-induced rat paw oedema test

The carrageenan-induced rat paw oedema test (Green and Green, 1971) was used to evaluate the *in vivo* acute anti-inflammatory activity of the samples of *C. citratus.* Diclofenac sodium was used as reference drug. The animals were divided into eight groups (n = 6): carrageenan control group received water 0.5 μL/g p.o., positive control group received diclofenac sodium 10 mg/kg i.p. and test groups received orally the extract at the doses of 34.12 mg/kg (CcE D'1) and 68.24 mg/kg (CcE D'2), the flavonoid-rich fraction at the doses of 3.71 mg/kg (CcF D'1) and 7.42 mg/kg (CcF D'2), and the tannin-rich fraction at the doses of 2.98 mg/kg (CcT D'1) and 5.96 mg/kg (CcT D'2).

Carrageenan control and test groups were dosed 1 h and positive control group was dosed 30 min before a subplantar injection of 0.1 mL of 1% solution of

carrageenan in saline, administered to the right hind footpad of each animal. The paw oedema volume was measured with a digital plethysmometer (model LE7500, Panlab, Barcelona, Spain) after carrageenan injection and then every hour during six hours. The anti-inflammatory effect was calculated 4 h after carrageenan administration and was expressed as percentage of oedema inhibition for the treated animals with respect to the carrageenan control group. The percentage of oedema inhibition was calculated according to the following equation:

$$\% \text{ oedema inhibition} = 1 - (Vt/Vc) \times 100$$

where Vt is the mean variation of the paw volume in rats treated with the sample or diclofenac sodium and Vc is the mean variation of the paw volume in the carrageenan control group.

2.5.2. The hot plate test

The hot plate test was used to evaluate the central analgesic activity of the samples by measuring the reaction time according to the method described by Eddy & Leimbach (Eddy and Leimbach, 1953). Morphine was used as reference drug. Mice were placed individually on a hot plate set at 55±1°C (model LE7406, PanLab, Barcelona, Spain). Reaction time was recorded when the animals licked their forepaws, shaked or jumped. The baseline was considered as being the reaction time before treatment. Then, the animals were divided into seven groups (n = 6-8): positive control group received morphine 10 mg/kg i.p. and test groups received orally the extract at the doses of 68.24 mg/kg (CcE D''1) and 136.48 mg/kg (CcE D''2), the flavonoid-rich fraction at the doses of 7.42 mg/kg (CcF D''1) and 14.84 mg/kg (CcF D''2), and the tannin-rich fraction at the doses of 5.96 mg/kg (CcT D''1) and 11.92 mg/kg (CcT D''2). Test groups were dosed 1 h and positive control group was dosed 30 min before placement of the animals on the hot plate for new recording of reaction time to temperature. Analgesic capacity was calculated by using the formula:

$$\% \text{ analgesic capacity} = 1 - (Tb/Tt) \times 100$$

where Tb is the mean reaction time recorded as baseline and Tt is the mean reaction time recorded after treatment with morphine or the samples.

2.5.3. The acetic acid-induced writhing test

The acetic acid-induced writhing test was carried out according to the method previously described by Collier *et al.* (Collier *et al.*, 1968). The test was used to evaluate the peripheral analgesic activity of the samples, employing diclofenac sodium as reference drug. Acetic acid 0.6% in saline was administered i.p., 0.1 mL/10 g b.w. The number of writhes – a response consisting of abdominal wall contractions followed by hind limb extension – was counted during continuous observation of the animals for 30 min, starting to count 5 min after the injection. The animals were divided into eight groups (n = 6-8): negative control group received water 0.5 μL/g p.o., positive control group received diclofenac sodium 10 mg/kg i.p. and test groups received orally the extract at the doses of 68.24 mg/kg (CcE D''1) and 136.48 mg/kg (CcE D''2), the flavonoid-rich fraction at the doses of 7.42 mg/kg (CcF D''1) and 14.84 mg/kg (CcF D''2), and the tannin-rich fraction at the doses of 5.96 mg/kg (CcT D''1) and 11.92 mg/kg (CcT D''2). Negative control and test groups were dosed 1 h and positive control group was dosed 30 min before the acetic acid injection. A significant reduction in the number of writhes by any drug was considered as a positive analgesic response. Analgesic capacity was calculated by using the formula:

$$\% \text{ analgesic capacity} = 1 - (Wt/Wc) \times 100$$

where Wt is the average number of writhes in test and positive control groups and Wc is the average number of writhes in the negative control group.

2.6. Histological analysis

A histological analysis was carried out in order to evaluate the toxicity of the *C. citratus* leaves essential oil-free infusion (CcE). The animals used in the carrageenan paw oedema test were sacrificed and the livers were collected, cut into transverse fragments (5 mm) and immersed in a 10% solution of formaldehyde for 24 h. The pieces were processed into paraffin and stored at -20°C. The fragments were cut in 4 μm pieces in a SHANDON (AS 325) microtome and stained with hematoxylin and eosin. The resulting images were obtained and observed using a NIKON (Eclipse E600) microscope.

2.7. Transmission electron microscopy (TEM)

Samples (2-3 mm) of liver collected from the untreated rats (control) and the rats treated with CcE at 34.12 mg/Kg (CcE D'1) and 68.24 mg/kg (CcE D'2) were fixed for 3 h at 4ºC in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) supplemented with 1 mM calcium chloride. Following rinsing in the same buffer, the samples were postfixed in 1% buffered osmium tetroxide for 1.5 h at 4 ºC and then in 1% aqueous uranyl acetate for 1h at room temperature. The samples were further dehydrated in a graded ethanol series (70%-100%) and embedded in Spurr's resin. Thin sections were obtained with a LKB Ultratome NOVA, ultramicrotome equipped with a diamond knife, conventionally stained with uranyl acetate and lead citrate and observed in a JEOL JEM-100 SX at 80 kV.

2.8. Statistical analysis

Data obtained from animal experiments were expressed as mean and standard error of the mean (mean±S.E.M.). Comparison between groups was done by One-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p<0.05$ were considered to be significant.

3. Results

3.1. Carrageenan-induced rat paw oedema test

Subplantar injection of the carrageenan solution into the right hind footpad of each rat led to a time-dependent increase in the paw volume. This increase was observed at 1 h and it was maximal at 4 h after administration.

Carrageenan-induced paw oedema volume was significantly reduced at the fourth hour by the administration of CcE D'1 and CcE D'2, CcF D'2, CcT D'2 and diclofenac sodium. The percentage of oedema reduction was 70.80% and 82.30% for CcE D'1 and CcE D'2, respectively, 59.00% for CcF D'2, 61.00% for CcT D'2 and 84.00% for positive control group. There was no significant difference between CcF D'1 or CcT D'1 and the negative control group (**Figure II.13**).

3.2. Hot plate test

Mice treated with morphine showed a marked increase in the reaction time with a maximum protective effect of 75.9%. However, the results reveal that mice treated with any of the samples did not show any significant increase in the baseline (**Figure II.14**).

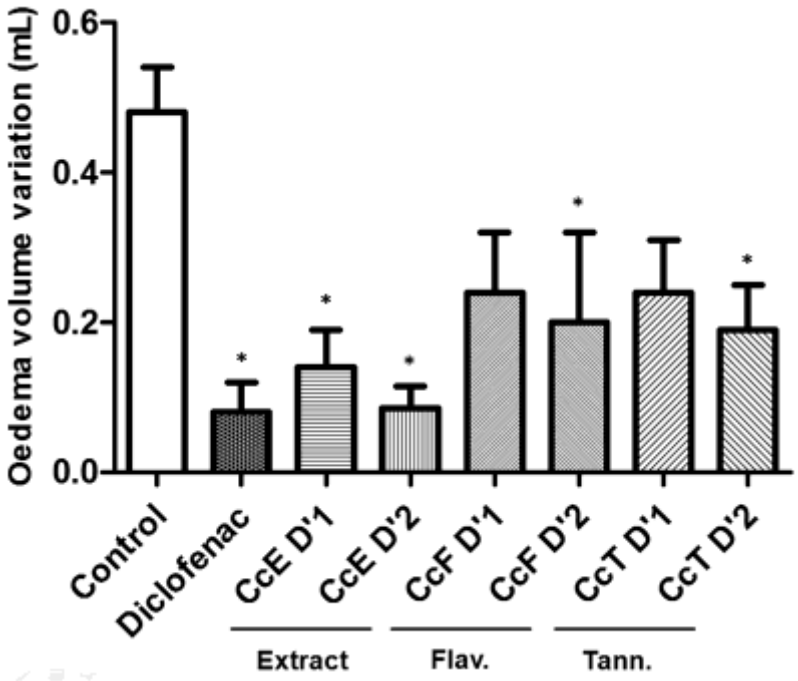

Figure II.13. Results from the *carrageenan-induced rat paw oedema test* were obtained after administering water 0.5 μL/g p.o. (negative control), diclofenac sodium 10 mg/kg i.p. (positive control) and *C. citratus* aqueous extract 34.12 mg/kg (CcE D'1) and 68.24 mg/kg (CcE D'2), *C. citratus* flavonoid-rich fraction 3.71 mg/kg (CcF D'1) and 7.42 mg/kg (CcF D'2) and *C. citratus* tannin-rich fraction 2.98 mg/kg (CcT D'1) and 5.96 mg/kg (CcT D'2) p.o. Each value is the mean±S.E.M. of 6 rats. Statistical differences between the treated and the negative control groups were determined by ANOVA followed by Bonferroni test. * $P <$ 0.05.

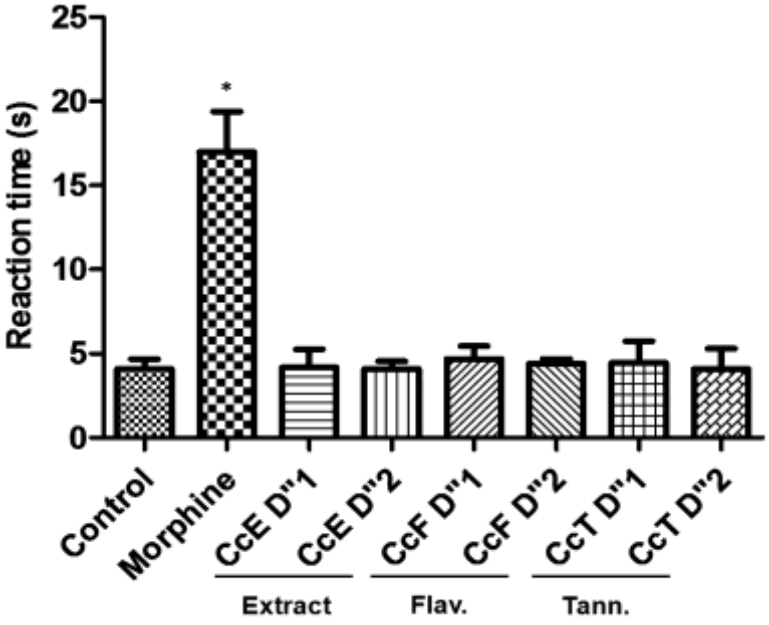

Figure II.14. Results from the *hot plate test* were obtained before and after administering morphine 10 mg/kg i.p. (positive control) and *C. citratus* aqueous extract 68.24 mg/kg (CcE D''1) and 136.48 mg/kg (CcE D''2), *C. citratus* flavonoid-rich fraction 7.42 mg/kg (CcF D''1) and 14.84 mg/kg (CcF D''2) and *C. citratus* tannin-rich fraction 5.96 mg/kg (CcT D''1) and 11.92 mg/kg (CcT D''2) p.o. Each value is the mean±S.E.M. of 6-8 mice/group. Statistical differences between the treated and the control groups were determined by ANOVA followed by Bonferroni test. * $P < 0.05$.

3.3. Acetic acid-induced writhing test

Mice treated with CcE D''2 showed a significant inhibition of the writhing response induced by acetic acid. The percent analgesic capacity was 27.5% (34.8 ± 8.1 writhes) and 57.0% (20.6 ± 9.7 writhes) for CcE D''1 and CcE D''2, respectively. Diclofenac sodium showed an analgesic capacity of 83.0% (13.0 ± 3.4 writhes). An important inhibition of the writhing response was also found for CcF D''2 and CcT D''2, with a percent analgesic capacity of 54.6% (21.8 ± 14.9 writhes) and 52.1% (23.0 ± 9.9 writhes), respectively. There was no significant difference between the lowest dose of flavonoids and tannins-rich fractions and the negative control group (48.0 ± 1.8 writhes), corresponding to 22.1% (37.4 ± 11.2 writhes) and 18.55% (39.1 ± 8.9 writhes) of percent analgesic capacity, respectively (**Figure II.15**).

3.4. Histological analysis

In **Figures II.16A**, **B** and **C** are represented the histological images of liver for control animals, animals treated with CcE single dosage (D'1) and double dosage (D'2), respectively, of the animals used in the carrageenan-induced rat paw oedema test. No differences were observed between the control and test groups, in both tested dosages. The images showed well shaped hepatic cells, with no signs of blood stasis in the extracellular medium or necrotic cells, revealing the absence of toxicity of the *C. citratus* leaves essential oil-free infusion.

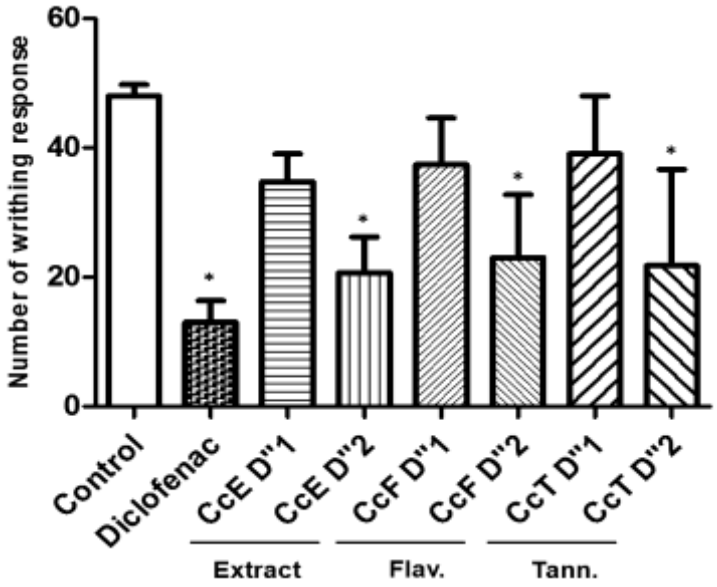

Figure II.15. Results from the *acetic acid-induced writhing test* were obtained after administering water 0.5 μL/g p.o. (negative control), diclofenac sodium 10 mg/kg i.p. (positive control) and *C. citratus* aqueous extract 68.24 mg/kg (CcE D''1) and 136.48 mg/kg (CcE D''2), *C. citratus* flavonoid-rich fraction 7.42 mg/kg (CcF D''1) and 14.84 mg/kg (CcF D''2) and *C. citratus* tannin-rich fraction 5.96 mg/kg (CcT D''1) and 11.92 mg/kg (CcT D''2) p.o. Each value is the mean±S.E.M. of 6-8 mice/group. Statistical differences between the treated and the control groups were determined by ANOVA followed by Bonferroni test. * $P <$ 0.05.

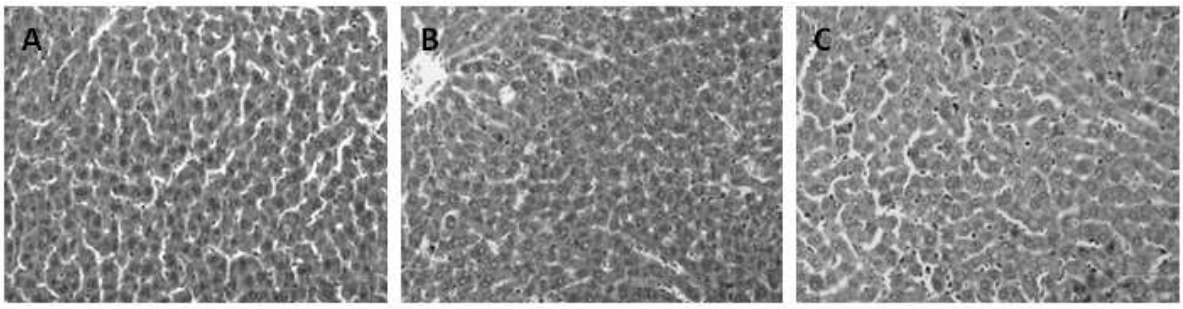

Figure II.16. Histological study of liver. Staining with hematoxylin (cell nuclei - blue) and eosin (cytoplasm - pink / red); 20x magnification in the original. (**A**) Negative control group; (**B**) Animals treated with CcE single dosage D'1(34.12 mg/kg); (**C**) Animals treated with CcE double dosage D'2 (68.24 mg/kg). Bar = 50 μm.

3.5. Liver cell ultrastructure

A general view of the ultrastructural organization of the rat liver parenchymal cells (hepatocytes) is shown in **Figure II.17A**. Briefly, hepatocytes are polygonal in shape with one, or sometimes two, large rounded nuclei containing one or two prominent nucleoli and a few dense masses of heterochromatin. In the whole cytoplasm, the most characteristic feature was the presence of a very high number of mitochondria showing a moderate electron-dense matrix and a reasonable number of electron-dense cristae (**Figures II.17A**, **B**). Both the smooth (SER) and rough endoplasmic reticulum (RER) were also abundant, the former appearing as irregularly shaped vesicular structures or short, branching tubules, and the latter as single or small groups of flattened cisternae often surrounding closely the mitochondria (**Figure II.17B**). A few lipid bodies of relatively small size, and a reasonable high number of peroxisomes with a homogeneous electron-dense matrix, generally containing a finely granular or crystalline core (nucleoid), were also frequently present (**Figures II.17A**, **B**). Secondary lysosomes, Golgi bodies and vesicles, and glycogen granules were sometimes seen. Adjacent hepatocytes were separated one from each other by a small intercellular space in which junctional complexes were present (**Figure II.17A**). The ultrastructure of hepatocytes in rats treated with both CcE D'1 (**Figure II.17C**) and CcE D'2 (**Figure II.17D**) did not differ significantly from that of hepatocytes in control rats (**Figure II.17A**). In general, the integrity of organelles, plasma membrane and junctional complexes remained intact and the

organelles retained the features characteristic of the control liver. Only in a few hepatocytes the mitochondria matrix sometimes appeared more electron-transparent (**Figure II.17C**) and with a more reduced number of cristae, and the lipid bodies could be larger (**Figure II.17D**) and more numerous in the cytosol.

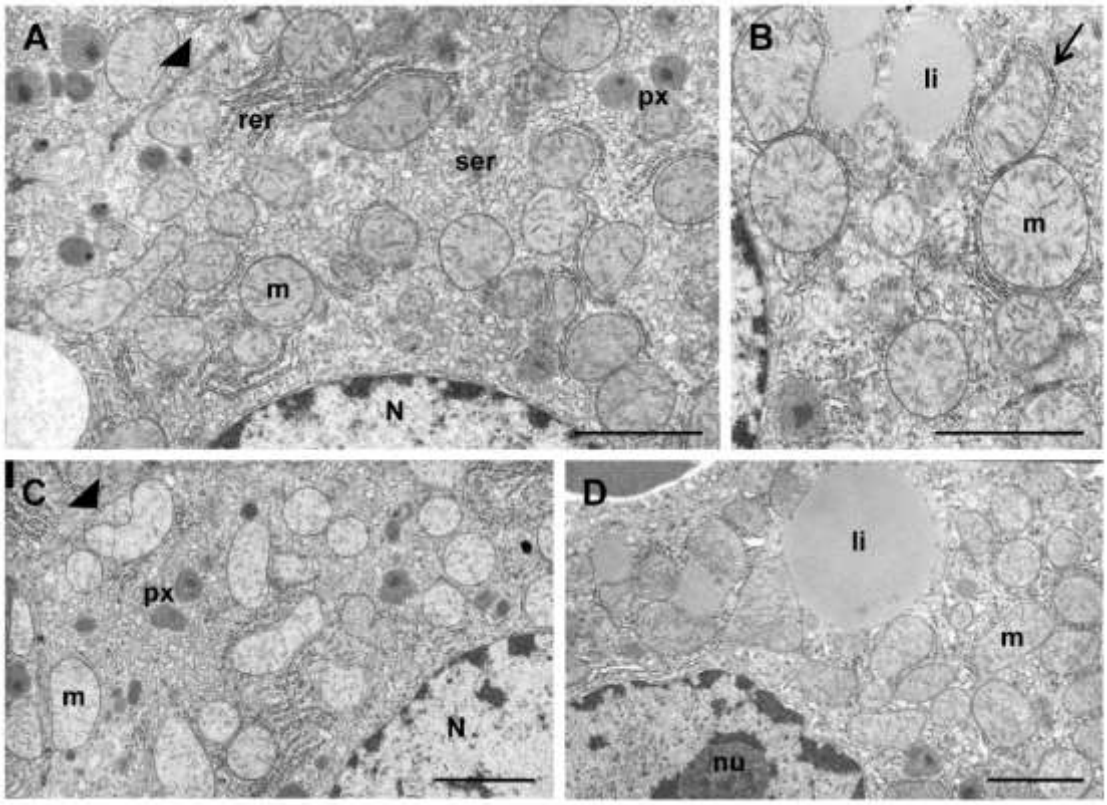

Figure II.17. Transmission electron micrographs of hepatocytes in control rats (**A** and **B**) and in rats treated with CcE D'1 (**C**) and with CcE D'2 (**D**). N - nucleus, nu - nucleolus, m - mitochondria, px - peroxisomes, rer - rough endoplasmic reticulum, ser - smooth endoplasmic reticulum, li - lipid bodies, arrow - rer cistern surrounding a mitochondria, arrowhead - plasma membranes of two adjacent hepatocytes. Bar = 1 μm.

4. Discussion and Conclusions

Anti-inflammatory and analgesic effects of many herbs have been related to their polyphenolic composition, mostly to flavonoids and tannins. Also, it has been reported that flavonoids such as luteolin produce significant antinociceptive and/or anti-inflammatory activities (Hosseinzadeh and Younesi, 2002). In regard to *C. citratus*, we have previously demonstrated (Figueirinha *et al.*, 2010) that the most active polyphenolic fractions of an essential oil-free infusion are those which held flavonoids and tannins and, when tested *in vitro*, CcE showed high anti-

inflammatory activity, inhibiting the NO production in dendritic cells. In the present study, anti-inflammatory and analgesic activities of an essential oil-free infusion of *C. citratus* leaves were evaluated to verify the claims made by folk medicine. Additionally, its flavonoid and tannin-rich fractions were tested, trying to better characterize their contribution for the pharmacological effects and also to deeper identify the responsible components of *C. citratus* for its traditional uses.

The *carrageenan-induced rat paw oedema assay* was performed to study the anti-inflammatory effect. This experimental model is commonly used for determining the acute phase of the inflammation because it is related with neutrophil infiltration, as well as the neutrophil-derived free radicals, prostaglandins and cytokines production (Cardoso *et al.*, 2003; Tanas *et al.*, 2010). Diclofenac sodium is widely used as a reference non-steroidal anti-inflammatory drug (NSAID), being commonly prescribed due to its anti-inflammatory and analgesic effects. This drug has the ability to reduce inflammation, swelling and pain by inhibiting either the release of arachidonic acid or the prostaglandin synthesis *in vivo* and *in vitro,* as well as by influencing the function of polymorphonuclear leukocytes *in vitro*, decreasing chemotaxis and free radical formation (Mahgoub, 2002; Ojewole, 2004).

Our present results indicate that the *C. citratus* infusion has anti-inflammatory activity, corroborating its traditional use. The observed effect for the highest dose was similar to that obtained by the reference NSAID used. The flavonoid and the tannin-rich fractions also exhibited anti-inflammatory activity at their highest dose, but when compared to the standard and the infusion, a lower inflammation inhibition was observed. Synergistic effects between the different phenolic compounds and/or the presence of other compounds in the extract can probably contribute for the CcE activity.

However, regarding the low doses that were used in the assay with the polyphenols, we may conclude about an important contribution of both flavonoids and tannins for the anti-inflammatory effect. This fact suggests that these polyphenols are significantly responsible for the overall effect observed with CcE. The mechanism behind these results is still unclear, but previous works point that

one of the major anti-inflammatory pathways modulated by these compounds might be the inhibition of NF-κB-regulated cytokines expression, probably from the scavenging capacity of reactive oxygen species, which modulates the activation of NF-κB pathway (Francisco *et al.*, 2011; Morgan and Liu, 2011). On the other hand, the same reactive species-scavenging ability may be related to the inhibition of polymorphonuclear leukocytes' action, such as neutrophils (Ojewole, 2004; Tanas *et al.*, 2010).

For the potential analgesic activity two different models were used to study both central and peripheral mechanisms of nociception. The *hot plate test* allows us to verify central analgesic effects while the *acetic acid-induced writhing test* evaluates peripheral analgesic effects of drugs (Queiroz *et al.*, 2010; Shang *et al.*, 2010).

The hot plate test is based on the animal behavior resulting from activation of the primary nociceptors by high temperature and, therefore, it is an excellent indicator of potential pharmacological drug effects. In the present study, morphine hydrochloride, a central-acting analgesic drug, which acts by activating opioid receptors (Eddy and Leimbach, 1953; Queiroz *et al.*, 2010; Ruangsang *et al.*, 2010), produced a pronounced inhibitory effect on the nociceptive response showed by the increase in mice reaction time; in contrast, the *C. citratus* infusion, its flavonoid- and tannin-rich fractions showed no antinociceptive activity in this test.

As mentioned above, the *acetic acid-induced writhing test* is useful to evaluate drugs with analgesic activity at a peripheral level of nociception. It is known that acetic acid acts indirectly by inducing the release of endogenous pro-inflammatory mediators such as histamine, serotonin, bradykinin, acetylcholine, P substance and prostaglandins involved in visceral inflammatory pain, by causing an increased vascular permeability (Queiroz *et al.*, 2010; Ruangsang *et al.*, 2010). This model is, therefore, sensitive to the action of NSAIDs (Dandawate *et al.*, 2012; Queiroz *et al.*, 2010). In this study, diclofenac sodium caused the inhibition of pain induced by acetic acid, and CcE, CcF and CcT showed also a significant reduction of the writhing response at the highest dose used. There were no relevant

differences in the inhibition of the number of writhes for the three samples, which leads us to conclude that flavonoids and tannins are the compounds that almost exclusively contribute for the *C. citratus* peripheral analgesic activity. This fact corroborates the published data about phenolic compounds exerting an antinociceptive effect *in vivo*, namely for tannins (DalBó *et al.*, 2005; Subarnas and Wagner, 2000) and flavonoids (Gorzalczany *et al.*, 2011; Valério *et al.*, 2009; Vidyalakshmi *et al.*, 2010).

This study reveals that CcE and its flavonoids and tannins are able to reduce paw oedema induced by carrageenan and the peripheral mechanism of pain, suggesting that their anti-inflammatory and analgesic effects may be related to the inhibition of the synthesis and release of various inflammatory mediators and to its antioxidant activity.

These results suggest that the CcE acts as anti-inflammatory and peripheral analgesic agent, still does not exhibit intrinsic central analgesic activity. Together, it can be inferred that the observed analgesic effect is likely associated with the inflammatory process, flavonoids and tannins having a significant contribution for these activities.

Liver plays a crucial role in the detoxification, modification and excretion of both endogenous and exogenous substances, including a variety of toxic substances. When exposed to toxic substances, hepatocytes show extensive alterations in the number, morphology and/or ultrastructure of organelles, such as mitochondria, endoplasmic reticulum, peroxisomes and lipid bodies (Lüde *et al.*, 2007). To investigate the potential for hepatotoxicity of *C. citratus* extracts in experimental animals *in vivo*, the liver tissue and ultrastructural organization of hepatocytes in control rats and in rats treated with CcE D´1 and CcE D´2 were examined. In the control rats, the cellular organization of hepatocytes was found to be very similar to that described by Baratta *et al.* (Baratta *et al.*, 2009). Following exposure to CcE D'1 and CcE D'2 no significant alterations were found in liver tissue and cell ultrastructure of the hepatocytes when compared with that of the untreated rats (control). Our results show that treatment with effective concentrations of the CcE induced no hepatotoxicity in rats and that, therefore,

the administration of CcE can be considered safe under the experimental conditions used.

In conclusion, our results support the traditional use of *C. citratus* infusion as an anti-inflammatory agent and suggest that CcE or its polyphenols, namely the flavonoid- and tannin-rich fractions, may be used to obtain a new anti-inflammatory medicine, effective in the treatment of inflammatory-related pathologies.

F. Polyphenols from *Cymbopogon citratus* leaves as topical anti-inflammatory agents

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Abstract

Ethnopharmacological relevance: A variety of plant polyphenols have been reported to have anti-inflammatory, frequently associated with erythema, oedema, hyperplasia, skin photoaging and photocarcinogenesis. *Cymbopogon citratus* is a worldwide known medicinal plant, used in traditional medicine in inflammation-related conditions.

Aim of the study: In this work, the anti-inflammatory potential of *Cymbopogon citratus* (DC). Stapf (Poaceae) infusion (CcI) and its polyphenols as topical agents was evaluated *in vivo.*

Materials and Methods: The plant extract was prepared and its fractioning led two polyphenol-rich fractions: flavonoids fraction (CcF) and tannins fraction (CcT). An oil/water emulsion was developed with each active (CcI, CcF+CcT and diclofenac), pH and texture having been evaluated. Release tests were further performed using static Franz diffusion cells and all collected samples were monitored by HPLC–PDA. *In vivo* topical anti-inflammatory activity evaluation was performed by the carrageenan-induced rat paw oedema model.

Results: The texture analysis revealed statistically significant differences for all tested parameters to CcF+CcT, supporting its topical application. Release experiments lead to the detection of the phenolic compounds from each sample in the receptor medium and the six major flavonoids were quantified, by HPLC-PDA: carlinoside, isoorientin, cynaroside, luteolin 7-*O*-neohesperidoside, kurilesin A and cassiaoccidentalin B. The CcF+CcT formulation prompted to the higher release rate for all these flavonoids. CcI4%, CcI1% and CcF+CcT exhibited an oedema reduction of 43.18, 29.55 and 59.09%, respectively.

Conclusions: Our findings highlight that CcI, containing luteolin 7-*O*-neohesperidoside, cassiaoccidentalin B, carlinoside, cynaroside and tannins have a potential anti-inflammatory topical activity, suggesting their promising application in the treatment of skin inflammatory pathologies.

Keywords: *Cymbopogon citratus*; lemongrass; topical application; anti-inflammatory; *in vivo* studies; drug release.

1. Introduction

Erythema, oedema, sunburn, hyperplasia, immunosuppression, skin photoaging and photocarcinogenesis are some of the causes of adverse skin reactions. All of these pathological conditions have in common a key trigger: inflammation (Yaar and Gilchrest, 2007). Two representative pro-inflammatory enzymes, cyclooxygenase-2 (COX-2) (Kundu *et al.*, 2008) and inducible nitric oxide synthase (iNOS) (Chang *et al.*, 2011), are aberrantly over-expressed in skin inflammatory conditions. Abnormally elevated expression of these enzymes as well as the NF-κB and IKKβ activation have been implicated in skin tumor promotion.

A wide variety of plant polyphenols has been reported to possess anti-inflammatory, immunomodulatory and antioxidant properties being among the most promising group of compounds that can be exploited as ideal chemopreventive agents for a variety of skin disorders in general and skin cancer in particular; especially flavonoids and tannins have been reported to exert pronounced anti-inflammatory and anti-carcinogenic effects both *in vitro* and *in vivo* (Das *et al.*, 2008; Singh and Katiyar, 2013).

Cymbopogon citratus (DC.) Stapf (Poaceae), commonly known as lemongrass, is a tropical perennial shrub originated from the Southeast Asia. In African and Latin American countries, this herb is highly consumed as an aromatic and pleasant-tasting herbal drink (Adeneye and Agbaje, 2007). Aqueous extracts of dried leaves are used in folk medicine for the treatment of several inflammation-based pathologies (Shah *et al.*, 2011). This plant is reported to possess antifungal, mosquito repellent, insecticidal, anti-diabetic, anti-septic, anti-mutagenic and anti-carcinogenic activities as well as anti-inflammatory (Francisco *et al.*, 2011; Garcia *et al.*, 2015). The antioxidant and radical scavenging activities of hydrophilic extracts of *Cymbopogon citratus* have been reported by several authors and related to its polyphenols (Campos *et al.*, 2014; Orrego *et al.*, 2009; Roriz *et al.*, 2014). In recent years, our group identified several compounds from lemongrass such as luteolin and apigenin glycosides and condensed tannins, respectively, strongly contributed to the anti-inflammatory and antioxidant properties of CcI (Costa *et al.*, 2015; Figueirinha *et al.*, 2010; Francisco *et al.*, 2011, 2013). Moreover, we

recently disclosed the anti-inflammatory activity of CcI *in vivo* after oral administration (Garcia *et al.*, 2015).

However, CcI topical anti-inflammatory effect on skin has not been yet reported. In the current work, we have studied the *in vivo* topical anti-inflammatory activity of a *C. citratus* leaves infusion and polyphenol-enriched fractions prepared from this extract.

2. Materials and Methods

2.1 Chemicals and drugs

Ethanol, methanol and formic acid were purchased from Merck® (Darmstadt, Germany). Sephadex® LH-20 was purchased from Sigma-Aldrich® (Amersham, Sweden). Propylene glycol (≥99.5%) and oleic acid (>99%) were purchased from Fluka® (Steinheim, Switzerland). Stearic acid 70 extra pure was obtained from Scharlau® (Sentmenat, Spain) and glycerol (>99.5%) from José M. Vaz Pereira (Benavente, Portugal). Diclofenac sodium was purchased from Novartis Pharmaceuticals SA (Barcelona, Spain). κ-Carrageenan and triethanolamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ketamine (Ketalar®) was purchased from Parke-Davis, Pfizer (Seixal, Portugal).

2.2 Plant material, extract preparation and fractioning

Dry leaves of *C. citratus* were acquired from ERVITAL (Mezio, Castro Daire, Portugal). A voucher specimen was then 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 (Life Sciences Department, University of Coimbra, Portugal).

A lipid- and essential oil-free infusion was prepared (CcI) as previously described (Figueirinha *et al.*, 2008). Briefly, 150 ml of boiling water were added to 5 g of grinded dry leaves, the mixture being kept hot and left to stand for 15 min. Afterwards, the polyphenol-enriched fractions were obtained as described by Costa *et al.* (Costa *et al.*, 2015a). Briefly, CcI was fractionated by a Flash Chromatography® apparatus (Buchi Labortechnik AG) on a reverse phase

preparative C18 column Buchi® (150 × 40 mm; particle size 40 - 63 μm) (Flawil, Switzerland), eluted with aqueous methanol, in a discontinuous gradient: 5% (0-40 min), 5-10% (40-55 min), 10% (55-85 min), 10-15% (85-90 min), 15-25% (90-110 min), 25-50% (110-140 min), 50% (140-160 min), 50-80% (160-180 min), 80-100% (180-200 min) and 100% (200-220 min) at a flow rate of 3 ml/min. The HPLC profile was registered at 280 and 320 nm by the UV detector C-640 Buchi® (Flawil, Switzerland), and the data was acquired using the software ECOMAC® 0.238 (Prague, Czech Republic). Two fractions were selected: F1 (0-120 min) and F2 (120-220 min). F1 and F2 were then sub-fractionated separately on a Sephadex® LH-20 column (35 x 4 cm) using ethanol as mobile phase. All fractionation processes were monitored by HPLC and thin layer chromatography (TLC) for polyphenols, providing two major fractions: flavonoids fraction, CcF (yield 7.4%) and tannins fraction, CcT (yield 3.8%). These fractions were chosen because they contained the most promising groups of phenolic compounds contained in CcI (Costa *et al.*, 2015a; Francisco *et al.*, 2014, 2013, 2011). Both extract and fractions were freeze-dried and kept at -20ºC until use.

2.3 Phenolic profile

2.3.1 HPLC-DAD-ESI/MS^n

HPLC-DAD-ESI/MS^n analysis was performed in a Surveyor® liquid chromatograph hyphenated to a diode-array detector (DAD) (Surveyor®) and a Finnigan® LCQ mass spectrometer (San Jose, CA, USA) equipped with an API-ES ionization chamber. Separation was performed on an Spherisorb® ODS-2 C_{18} reverse phase column, 150 x 2.1 mm i.d. and particle size of 3 μm (Waters® Corporation, Milford, Massachusetts, USA) and a Spherisorb® ODS-2 guard cartridge C18 (10 x 4.6 mm i.d. and particle size of 5 μm; Waters® Corporation, Milford, Massachusetts, USA) at 25ºC, using 2% aqueous formic acid (A) and methanol (B) as mobile phase. The gradient profile used was 5-15% B (0-10 min), 15-30% B (10-15 min), 30-35% B (15-25 min), 35-50% B (25-35 min), 50-80% B (35-40 min), 80% B (40-60 min), isocratically, at a flow rate of 0.2 mL/min. The

first detection was done with the PDA detector in a wavelength range of 200-600 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 MS^1 (*m/z* 160-1300), MS^2 of the most abundant ion in MS^1, and MS^3 of the most abundant ion in MS^2. Source voltage was 4.5 kV and capillary voltage and temperature were -10 V and 250ºC, respectively. Nitrogen was used as sheath gas at flow rate of 20 arbitrary units. The normalized energy of collision was 45%, using helium as collision gas.

2.3.2 HPLC-DAD

HPLC analyses were performed in a chromatograph GILSON equipped with a diode-array detector (DAD) (Gilson® Electronics SA, Villiers le Bel, France). The studies were carried out on a Spherisorb S5 ODS-2 column (250 x 4.6 mm i.d.; particle size, 5 μm; Waters® Corp., Milford, MA, USA) at 25ºC and a Nucleosil C18 guard cartridge (30 x 4 mm i.d.; particle size, 5 μm; Macherey-Nagel, Düren, Germany). A mobile phase of 5% (v/v) aqueous formic acid (A) and methanol (B) was used with a discontinuous gradient: 5–15% B (0–10 min.), 15–30% B (10–15 min.), 30–35% B (15–25 min.), 35–50% B (25–35 min.) and 50–80% B (35–40 min.), followed by an isocratic elution for 20 min., at a flow rate of 1 ml/min. An injection volume of 100 μl was used for all standards and samples. Chromatographic profiles were acquired in the wavelength range 200–600 nm, and recorded at 280 nm. Data treatment was carried out with Unipoint® 2.10 software (Gilson®). The quantification of the analytes was achieved through HPLC-DAD. The main validation parameters of the analytical method employed were in agreement with the international guidelines (FDA, 2013) and are summarized in **Table II.8**.

Table II.8. Validation parameters of the HPLC method employed for the quantification of phenolic compounds in samples (n = 3).

Standard	Concentration range (μg mL^{-1})	Calibration curve	R^2	$F_{calculated}$*	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Caffeic acid	0.50 – 10.00	y = 9957547x – 1980541	1	1.37	0.65	1.97
***p*-Coumaric acid**	0.50 – 10.00	y = 65289976x + 522431	1	3.19	0.11	0.32
Isoorientin	7.81 – 250.00	y = 1939071x – 4248388	1	2.87	0.13	0.40
Isovitexin	7.81 – 250.00	y = 19173001x + 1904129	1	3.15	0.11	0.33

*$F_{calculated}$ should be lower than 3.26 ($F_{tabulated}$ at 95% with 4 and 12 degrees of freedom).

2.4 Topical formulations

An oil/water emulsion was used to convey the extract and fractions. The excipients were previously screened (data not shown) in order to achieve the optimal formulation for the crude extract, yielding the following final formula: stearic acid (14%, w/w), triethanolamine (1.2%, w/w), glycerol (13.5%, w/w), propylene glycol (5%, w/w), oleic acid (10%, w/w) and water (qs 100%, w/w). Five formulations were prepared: placebo, 1% (w/w) diclofenac, 4% (w/w) extract (CcI4%), 1% extract (CcI1%) and 1% polyphenol-enriched fraction containing 0.66% (w/w) CcF + 0.34% (w/w) CcT (CcF+CcT). Flavonoid-enriched and tannin-enriched fraction concentrations were calculated, taken in consideration the relative proportion of each fraction in the extract.

2.5 Formulation assessment

2.5.1 pH Evaluation

In order to verify the chemical stability of all tested formulations, the pH was monitored, using a Five Easy FE20 pH reader (Mettler Toledo®, Greifensee, Switzerland). Aliquots of the samples were measured at 0, 3, 4, 7, 10 and 15 days.

2.5.2 Texture profile analysis

Texture characteristics of the formulations were inspected through a Texture Analyzer TA.XT Plus (Stable Micro Systems Ltd., Surrey, UK). TPA mode was performed using an analytical probe (P/10, 10 mm Delrin) which was twice

depressed into the sample at a defined rate (5 mm/s) to a desired depth (15 mm), allowing 15 sec of delay between consecutive compressions, as previously described by Vitorino and co-workers (Vitorino *et al.*, 2013).

Six replicates were performed at 32 ºC, the temperature at skin surface, in the temperature controlled Peltier Cabinet for each formulation. Data analysis was carried out through the Texture Exponent 3.0.5.0 software package of the instrument.

2.6 *In vitro* release studies

In vitro release studies were performed using static Franz diffusion cells (PermeGear, Inc., PA, USA) with a diffusion area of 0.636 cm^2 and a receptor compartment of 5 ml. A dialysis cellulose membrane (MWCO~12000, avg. flat width 33 mm, D9652, Sigma-Aldrich), as artificial membrane, was placed between both compartments and a receptor solution of phosphate buffer saline (PBS, pH = 7.4) with 30% ethanol (v/v) to warrant sink conditions was used. This receptor phase was stirred at 600 rpm and maintained at 37 ± 0.5 ºC by a thermostatic water pump, assuring a temperature of 32 ºC at the surface, thus mimicking human skin conditions. The samples (400 µl) were applied in the donor compartments, and occluded with Parafilm® to prevent evaporation. The experiments were carried out over a period of 48 h, at several time points, 300 µl aliquots were collected of the receptor compartment, replacing this volume by fresh medium. All aliquots were analyzed and the major flavonoids quantified by HPLC-DAD.

2.7 *In vivo* assays

2.7.1 Animals

Male Wistar rats weighing 120-220 g and male mice weighing 20-30 g were acquired from Charles River (Barcelona, Spain). The animals were maintained with food and water *ad libitum* and kept at 22 ± 1ºC with controlled 12 hour light/dark cycle at the Faculty of Pharmacy (University of Coimbra). The animals were allowed to adapt to the laboratory for 7 days before testing and fasted with free

access to water at least 24 h prior to experiments. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Directive 2010/63/EU.

2.7.2 Carrageenan-induced paw oedema assay

The carrageenan-induced rat paw oedema test (Green and Green, 1971) was used to evaluate the topical acute anti-inflammatory activity of the samples. Diclofenac sodium was used as reference drug. The animals were divided into five groups (n = 6): carrageenan control group received negative control formulation; positive control group received 1% diclofenac sodium formulation; and test groups received CcI4%, CcI1% and CcF+CcT, respectively. All formulations were administrated in an amount of 1 ml. Negative control and test groups were dosed 1 h and positive control group was dosed 30 min before a subplantar injection of 0.1 ml of 1% (w/v) solution of carrageenan in saline, administered to the right hind footpad of each animal. The paw oedema volume was measured with a digital plethysmometer (model LE7500, Panlab, Barcelona, Spain) after carrageenan injection and then every hour during six hours, for the animals of control group. For the test groups, the anti-inflammatory effect was calculated 4 h (t_{max}) after carrageenan administration and was expressed as percentage of oedema inhibition in the treated animals with respect to the carrageenan control group. The percentage of oedema inhibition was calculated according to the following equation: % oedema inhibition = 1 - (Vt/Vc) x 100, where Vt is the mean variation of the paw volume in rats treated with the sample or diclofenac sodium and Vc is the mean variation of the paw volume in the carrageenan control group.

2.8 Statistical analyses

Data obtained from animal experiments were expressed as mean and standard error of the mean (mean±S.E.M.). Comparison between groups was carried out by One-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p<0.05$ were considered to be statistically significant. For the remaining data, the statistical package Windows® MS Excel 2007 was used.

3. Results and Discussion

3.1 Phenolic profile

In order to characterize CcI, the phenolic profile was achieved by HPLC-DAD (**Figure II.18**). The detected phenolic compounds were identified by HPLC-DAD-ESI/MS^n (**Table II.9**). In order to better characterize CcI and CcF, some marker compounds were quantified (**Table II.10**). The markers were chosen because they are the most abundant bioactive flavonoids of lemongrass infusion. CcT have been already characterized by our group (Costa *et al.*, 2015a).

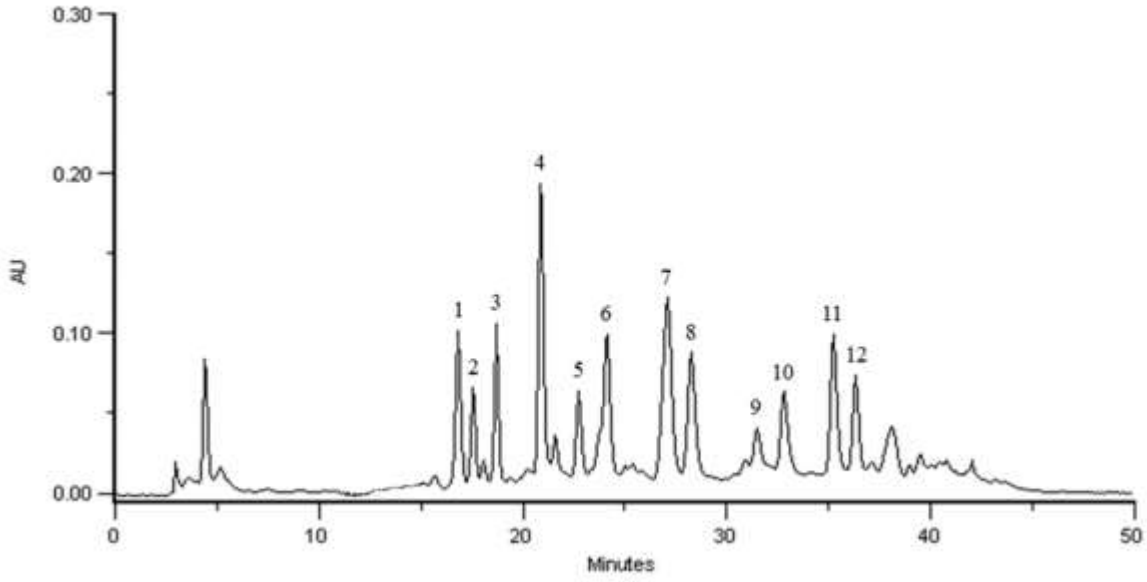

Figure II.18. HPLC fingerprint of CcI, recorded at 280 nm, by HPLC-DAD.

Table II.9. Phenolic compounds identification of *Cymbopogon citratus* infusion, by HPLC-DAD-ESI/MS^n (cont.).

Peak	Identification	λmaxima (nm)	$[M-H]^-$ (*m/z*)	MS^2 [*m/z* (rel. abundance, %)]	MS^3 [*m/z* (rel. abundance, %)]
1	Feruloylquinic acid	290, 325	367	193(100)	149(100)
2	Neochlorogenic acid	290, 326	353	191(100), 179(10), 109(2)	191(60), 173(100), 93(68)
3	Chlorogenic acid	290, 326	353	191(100), 179(60), 109(10)	191(60), 173(100), 93(70)
4	*p*-Coumaric acid	295, 310	163	-	-
5	Carlinoside	258sh, 272, 329	579	519(2), 489(32), 459(100), 399(25)	441(26), 399(100), 369(80)
6	Isoschaftoside	272, 331	563	545(30), 503(79), 473(100), 443(76), 383(43), 353(40)	383(15), 353(100)
7	Isoorientin	258sh, 269, 348	447	429(35), 387(2), 357(100), 327(75), 285(1)	357(100), 339(60), 297(28), 285(10)
8	Isoorientin 2"-*O*-rhamnoside	258sh, 269, 350	593	575(2), 473(100), 429(60), 357(24), 339(10)	399(2), 327(100), 298(20), 285(2)
9	Luteolin 7-*O*-neohesperidoside	258sh, 268, 347	593	447(100), 327(18), 285(74), 199(1)	327(2), 285(100)
10	Kurilensin A	258sh, 270, 350	563	545(19), 503(13), 473(100), 459(16), 399(34), 369(16)	399(100), 369(73)
11	Luteolin 6-*C*-pentoside	258sh, 272, 348	417	399(22), 357(100), 327(35)	339(100), 327(16), 311(20), 297(87), 285(17)
12	Cassiaoccidentalin B	258sh, 270, 348	575	531(21), 513(10), 429(21), 411(100), 367(42), 337(12)	411(2), 393(12), 364(90), 337(100), 311(12), 285(8)

Table II.10. *Cymbopogon citratus* marker compounds quantified in CcI and CcF by HPLC-DAD.

Peak	Compound	Measured concentration mean ± SD (μg mL^{-1})	
		CcI	CcF
1	3-Feroylquinic acid[a]	3.82 ± 0.04	n.d.
3	Chlorogenic acid[a]	4.40 ± 0.30	n.d.
4	*p*-Coumaric acid	8.01± 0.21	n.d.
6	Isoschaftoside[b]	11.35 ± 0.23	563.28 ± 0.05
7	Isoorientin[c]	52.02 ± 0.54	688.53 ± 0.21
8	Cynaroside[c]	23.45 ± 0.26	462.42 ± 0.18
10	Luteolin 7-*O*-neohesperidoside[c]	11.12 ± 0.13	180.59 ± 0.09
11	Kurilensin A[c]	26.48 ± 0.29	535.69 ± 0.12
12	Cassiaoccidentalin B[c]	13.78 ± 0.16	316.21 ± 0.40

[a]Concentration expressed in μg equivalents of caffeic acid per mL; [b]Concentration expressed in μg equivalents of isovitexin per mL; [c]Concentration expressed in μg equivalents of isoorientin per mL.

3.2 Formulation assessment

3.2.1 pH measurement

The pH values are presented at **Figure II.19**. All formulations were stable during the 15 days period. With the exception of CcI4%, all formulations exhibited a pH value very similar to that of the placebo and compatible with a topical skin application.

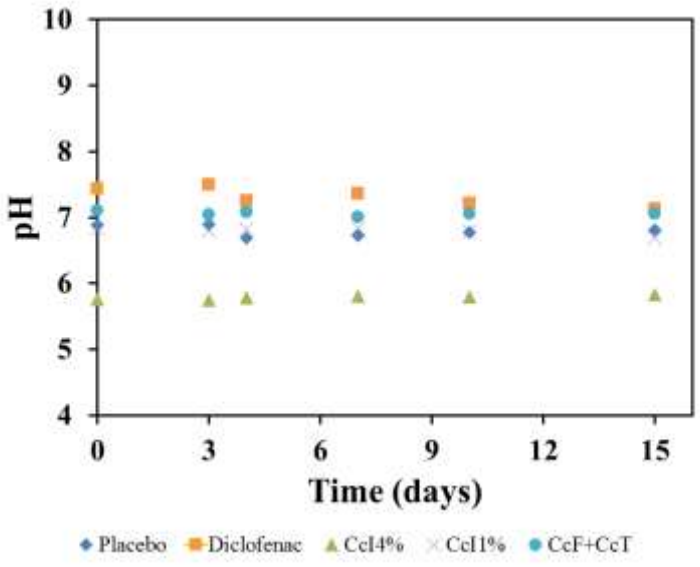

Figure II.19. pH measurement values of the topical formulations over time.

3.2.2 Texture evaluation

In order to assess interactions between formulation components and their impact upon the respective biological performance, mechanical properties of the different formulations were monitored through the texture profile analysis (TPA). From the force-time curve obtained, the following mechanical parameters can be extracted (see **Figure II.20**): hardness, which corresponds to the maximum peak force during the first compression cycle; compressibility, i.e., the work required to deform the sample during the first compression of the probe and estimated from area under the force–time curve 1 (AUC_1); adhesiveness, the work required to overcome the attractive forces between the surface of the sample and the surface of the probe and calculated from the negative force area for the first compression cycle (AUC_2); cohesiveness, determined by the ratio of the area under the force–time curve produced on the second compression cycle to that on the first compression cycle, where both compressions are separated by a defined recovery period; elasticity, obtained from the ratio of the time required to achieve maximum structural deformation on the second compression cycle to that on the first compression cycle. The results are presented in **Table II.11**. Regarding hardness, which expresses the ease with which the cream is applied on the skin, an increase was observed when the extract was included. Note that small values of hardness are often associated with a large relative uncertainty. Maximal values were obtained for the CcF+CcT formulation (9.0 g), while no difference was obtained between CcI1% and CcI4% formulations (1.0 and 0.9 g, respectively). An increased force /unit time required for compression and, subsequently, increased hardness is related to the increased viscosity of the formulation. However, according to previous studies (Vitorino *et al.*, 2013), the hardness values obtained in this work are acceptable for skin application.

Concerning to compressibility and adhesiveness, again higher values were obtained for CcF+CcT formulation (10.0 and -16.0 g.sec, respectively), followed by CcI1% formulation (4.0 and -8.0 g.sec, respectively). These parameters are correlated to the spreadability of the cream on the skin surface and bioadhesion, respectively. Despite the increase in compressibility values, it still reflects an easy

application, which is combined with a higher adhesion. This is consistent with the results obtained from release studies (see *In vitro* release studies), where the higher release rate observed is possibly favored by a better contact with the skin.

In relation to the elasticity, which is defined as the rate at which the deformed sample returns to its original condition after the removal of the deforming force, the same trend was observed. It is described that lower quantitative values of elasticity obtained from TPA indicate larger gel elasticity. Although the higher elasticity value corresponds to the CcF+CcT formulation (0.8), the cream has an acceptable elasticity compared to previous literature findings (Vitorino *et al.*, 2013).

Regarding cohesiveness, it provides information on the structural reformation following cream application, and usually, a high value is associated with a full structural recovery (Senyiğit *et al.*, 2011). The analysis of the present results revealed statistically significant differences in this parameter for CcF+CcT, supporting its topical application.

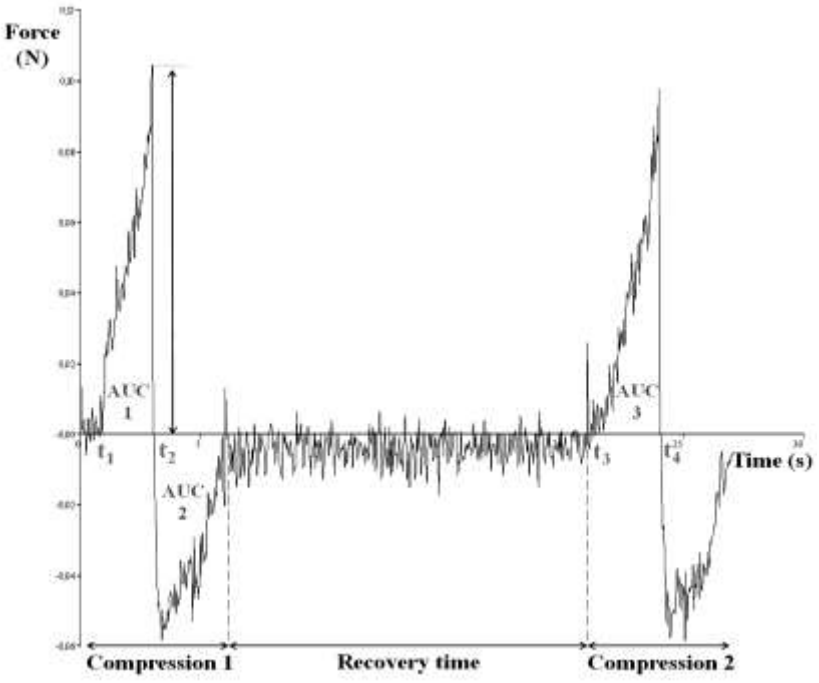

Figure II.20. Graphical output from texture profile analysis (H=hardness; AUC_1= compressibility; AUC_2= adhesiveness; AUC_3/AUC_1 corresponds to the cohesiveness; elasticity is given by time diff 3:4/time diff 1:2).

Table II.11. Mechanical properties of the formulations extracted from the TPA mode.

Formulations	Compressibility (g.sec)	Hardness (g)	Adhesiveness (g.sec)	Elasticity	Cohesiveness
Negative Control	0.7 ± 0.7	0.4 ± 1.6	-2.0 ± 3.0	0.3 ± 0.1	0.6 ± 0.3
Diclofenac	4.6 ± 0.9*	0.7 ± 1.7	-6.1 ± 3.9	0.8 ± 0.2*	0.9 ± 0.1*
CcI4%	1.2 ± 0.3	0.9 ± 1.8	-2.0 ± 2.6	0.3 ± 0.4	0.6 ± 0.4
CcI1%	4.0 ± 1.3*	1.0 ± 3.0	-8.0 ± 2.0*	0.5 ± 0.1*	0.8 ± 0.2
CcF+CcT	10.0 ± 4.0*	9.0 ± 5.1*	-16.0 ± 4.1*	0.8 ± 0.2*	0.9 ± 0.1*

Results are indicated as a mean of six replicates ± standard deviation (*p<0.05 *vs.* negative control).

3.3 *In vitro* release studies

Release profiles for the formulations containing 4% (w/w) and 1% (w/w) of CcI, and CcF+CcT are shown in **Figure II.21**. The cumulative amount of the major flavonoids: 6-*C*-β-glucopyranosyl-8-*C*-α-arabinopyranosyl-luteolin (carlinoside), 6-*C*-β-glucopyranosyl-luteolin (isoorientin), 7-*O*-β-glucopyranosyl-luteolin (cynaroside), luteolin 7-*O*-neohesperidoside, 6-*C*-(2''-*O*-α-rhamnopyranosyl)-α-arabinofuranosyl)-luteolin (kurilensin A) and 6-*C*-(2''-*O*-α-rhamnopyranosyl)-(6-deoxy-α-ribo-glucopyranosyl)-luteolin (cassiaoccidentalin B) – was determined by HPLC-DAD, using isoorientin as standard. Release profiles revealed a burst effect after 30 minutes of formulation application, followed by a sustained release throughout the period of the experiment. This suggests its efficacy for application on skin, particularly for the treatment of inflammation disorders. It can be also observed that all flavonoids were generally released to a higher extent in the formulation containing CcF+CcT, even with a lower loading in comparison to the other formulations. On the other hand, CcI1% exhibited higher release values, for all detected flavonoids, than CcI4%. This fact may be explained by the different matrix effect of each sample in the semisolid formulation.

These results are in good agreement with the mechanical properties previously referred. Note that for the CcF+CcT formulation higher values of

adhesion, hardness and compressibility were obtained, thus suggesting the increased release observed, which might possibly be a result of a closer contact with the stratum cornea. A thorough examination of the compound behavior reveals that luteolin 7-*O*-neohesperidoside, cassiaoccidentalin B and carlinoside generally, exhibited higher release rates. These findings may be ascribed to their different log P (-0.6, -0.3, -2.5, respectively *vs*. -0.2 and 0.5 for isoorientin and cynaroside, respectively) and high polar surface area (PSA) (245, 233, 267 Å, respectively *vs*. 197 and 186 Å for isoorientin and cynaroside, respectively) (National Center for Biotechnology Information, n.d.). Moreover, the use of an oil/water emulsion itself further contributes to an increase of the release of the referred compounds.

3.4 Carrageenan-induced paw oedema assay

The results show a statistically significant reduction of oedema volume for CcI4%, CcF+CcT and diclofenac formulations, compared to the placebo (**Figure II.22**). There was a reduction in the volume of the oedema induced by carrageenan of 43.18% for CcI4%, 29.55% for CcI1%, 59.09% for CcF+CcT formulation and 65.91% for the positive control, respectively. These results confirm the anti-inflammatory activity of the *C. citratus* infusion topical formulation, as well as the resulting formulation combining the fractions enriched in flavonoids and tannins, suggesting that the major contribution to the observed activity may rely on these polyphenols. These results well correlate with the *in vitro* experiments, which revealed that the CcF+CcT was the formulation that prompted to the highest amount of released compounds. More recently, the luteolin derivatives, present in the tested samples, have been co-related with the inhibition of inflammatory mediators like iNOS and cytokines TNF-α, IL-1β and IL-6 (Francisco *et al.*, 2014).

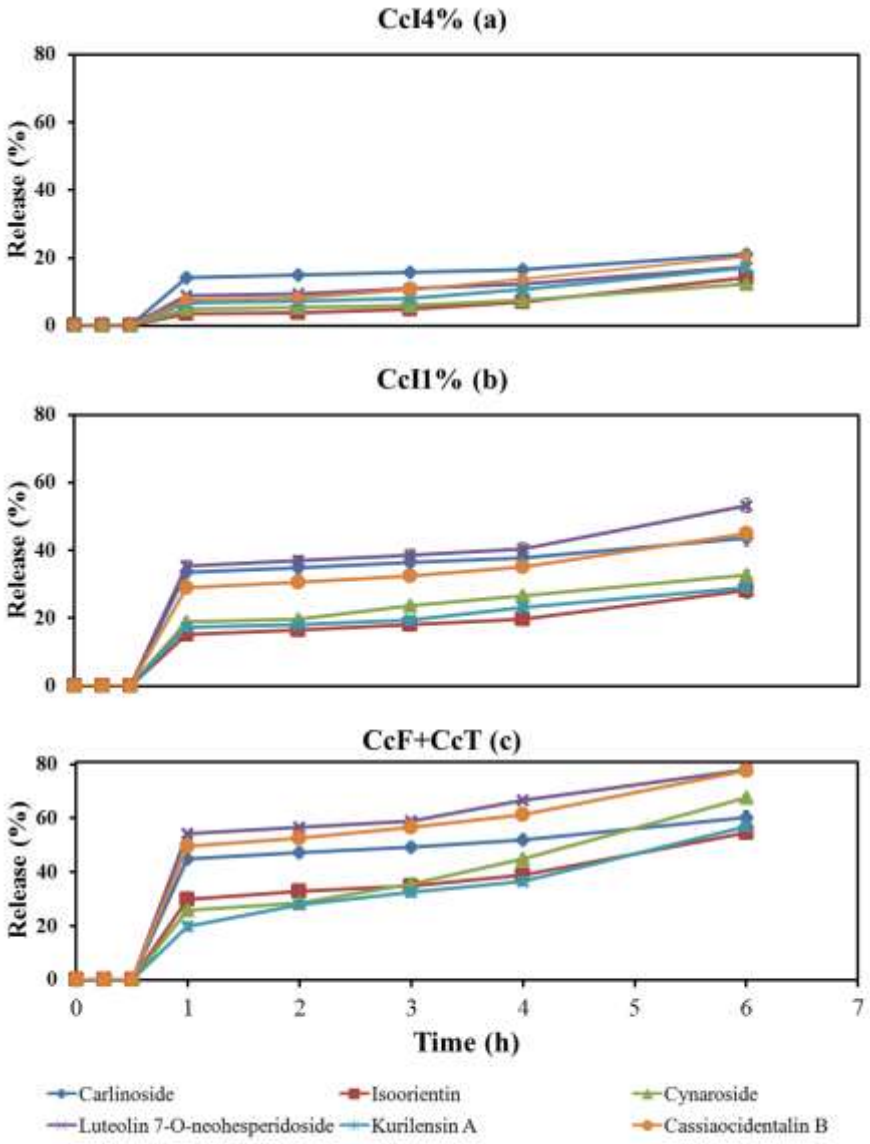

Figure II.21. Franz diffusion cells release profiles of carlinoside, isoorientin, cynaroside, luteolin 7-*O*-neohesperidoside, kurilensin A and cassiaoccidentalin B for the CcI4% (**a**), CcI1% (**b**) and CcF+CcT (**c**) formulations.

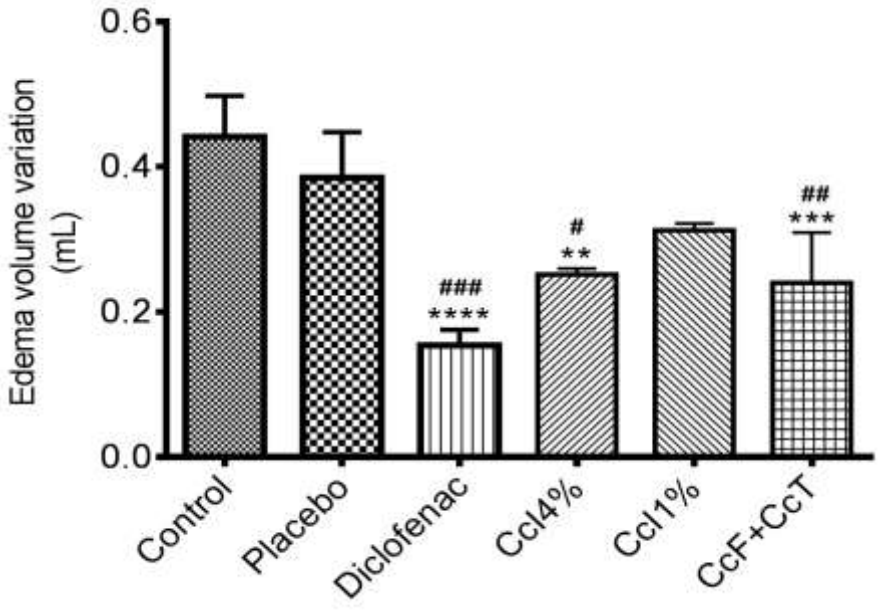

Figure II.22. Effect of topical application of the placebo, positive control (diclofenac sodium, 1%), CcI4%, CcI1%, CcF+CcT formulations on the oedema volume induced by 1% carrageenan, 4 h after inflammation induction. Each result represents the mean ± SEM (n = 6/group). **p<0.01, ***p<0.001 and ****p<0.0001, compared with the control group. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001, compared with the placebo group.

4. Conclusions

In conclusion, our results suggest that CcI, and its polyphenols, tannins and flavonoids, mainly, luteolin 7-*O*-neohesperidoside, cassiaoccidentalin B, carlinoside and cynaroside, may contribute to the topical anti-inflammatory effect, observed in this work. In light of this study, these polyphenols could be active constituents to integrate in a new anti-inflammatory agent, effective for the treatment of skin inflammatory-related pathologies.

G. Pharmacokinetics of *Cymbopogon citratus* infusion in rats after single oral dose administration

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Abstract

Cymbopogon citratus, commonly known as lemongrass, is a tropical herb used in worldwide traditional medicine for centuries. Studies previously conducted by our team demonstrated its antioxidant and anti-inflammatory effects, and recently, the anti-inflammatory potential was also observed *in vivo*. However, little is known about its pharmacokinetics. The current study aimed at obtaining, for the first time, the pharmacokinetic profile of lemongrass infusion after a single dose oral administration to rats.

All *in vivo* experimental procedures were approved by the Portuguese Veterinary General Division. Male Wistar rats were administered with a single oral dose of lemongrass infusion (68.24 mg/kg) and aliquots of plasma were collected at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h post-dosing. Liver and kidney samples were collected at 1, 2, 4, 8 and 24 h post-dosing. Plasma and tissues homogenates were pre-treated by a solid-phase extraction procedure and luteolin (LUT), luteolin 7-*O*-glucuronide (L7G), chrysoeriol (CHR), diosmetin (DIO) and luteolin 3'-*O*-sulphate (L3'S) were quantified employing a RP-HPLC-PDA method. The mean concentration-time profiles obtained were analyzed by a non-compartmental pharmacokinetic analysis using the WinNonlin®. The plasma and tissues C_{max}, T_{max}, $T_{1/2}$, $AUC_{(0\text{-}t)}$, $AUC_{(0\rightarrow\infty)}$ and $MRT_{(0\rightarrow\infty)}$ were determined.

The pharmacokinetic studies revealed the presence of LUT, L7G, CHR, DIO and L3'S. L7G and L3'S were rapidly detected, with maximum plasma concentration at 30 min after oral administrations. The concentration-time profile of liver samples evidenced compounds undetected in plasma: LUT, CHR and DIO. L7G, CHR and L3'S were detected in the liver from the first hour and stayed in the tissue until at least 24h. The kidney concentration-time profile revealed the presence of the same compounds detected in plasma.

The pharmacokinetic analysis showed that the compounds present in lemongrass infusion are not detected in plasma, liver or kidneys. On the other hand, L7G and L3'S were the major metabolites found in plasma and tissues, suggesting that lemongrass polyphenols are promptly metabolized *in vivo* and their

metabolites may be the ones responsible for the anti-inflammatory activity of *C. citratus*, when orally administered.

Keywords: *Cymbopogon citratus*, lemongrass, flavonoids, pharmacokinetics, *in vivo*.

1. Introduction

Cymbopogon citratus (DC.) Stapf is a tropical herb of the Poaceae family from Southeast Asia, commonly known as lemongrass. Studies previously conducted demonstrated its hypoglycemic, hipolypidemic, anxiolytic, sedative, antioxidant and anti-inflammatory effects (Adeneye and Agbaje, 2007; Campos *et al.*, 2014; Orrego *et al.*, 2009; Salim *et al.*, 2014; Tapia *et al.*, 2007). Some researchers have studied diverse-origin samples of lemongrass regarding their phenolic composition and antioxidant and anti-inflammatory properties (Figueirinha *et al.*, 2008; Roriz *et al.*, 2014; Soares *et al.*, 2013; Tiwari *et al.*, 2010). In the past years, the phenolic composition of lemongrass infusion was described: 3-feroylquinic acid, neochlorogenic acid, chlorogenic acid, *p*-coumaric acid, carlinoside, isoschaftoside, isoorientin, cynaroside, veronicastroside, luteolin 7-O-neohesperidoside, kurilensin A and cassiaoccidentalin B (Costa *et al.*, 2015b). We also demonstrated that flavonoids, such as luteolin and apigenin glycosides, and tannins strongly contributed to antioxidant and anti-inflammatory properties of an essential oil-free infusion from lemongrass (Costa *et al.*, 2015a; Figueirinha *et al.*, 2010, 2008; Francisco *et al.*, 2014, 2013, 2011). Very recently, the anti-inflammatory and analgesic potential of lemongrass infusion was also revealed *in vivo* by our research group (Garcia *et al.*, 2015). These activities were ascribed to mono- and polymeric flavonoid-enriched fractions of the crude extract, in which flavones *O*- and mainly *C*-glycosides and flavanic tannins, respectively, were identified, suggesting, therefore, a straight relationship between these phenolic compounds and their properties aforementioned.

Biological effects of flavonoids imply them to be bioavailable and to effectively reach target tissues. Thus, a number of studies have been conducted in order to investigate the metabolism of naturally occurring flavonoids, focusing particularly on the role of the gut microflora in the metabolic transformation of flavonoids (Marín *et al.*, 2015; Rice-Evans, 2004). Accordingly, following the ingestion of flavonoid *O*-glycosides and before their intestinal absorption, the corresponding sugar moieties (as in quercetin 3-*O*-glucoside) are cleaved from the phenolic backbone by enzymes expressed at the enterocyte membrane such as

lactase phlorizin hydrolase (LPH) or cytosolic enzymes such as *β*-glucosidase (CBG), which hydrolyze glycosylated flavonoids, allowing the formed aglycones to enter epithelial cells by passive diffusion (Gee *et al.*, 2000). However, flavonoids linked to a rhamnose moiety must reach the colon to be hydrolyzed by the *α*-rhamnosidases secreted by the colon microbiota (as *Bifidobacterium dentium*), in order to be absorbed (Marín *et al.*, 2015). Once the final derivative or the aglycone have been absorbed, they suffer phase II metabolism at enterocyte level; these reactions include methylation at C3' or C4' by catechol-*O*-methyltransferase (COMT), sulfation at C3', C4', C5, or C7 by sulfotransferases (SULT) and/or glucuronidation by UDP-glucuronosyltransferases. Afterwards, these products enter the blood stream by the portal vein, reaching the liver, where they may be subjected to more phase II metabolism, hence becoming conjugated and transported to the bloodstream again until they are secreted in urine (Chen *et al.*, 2011; Ouzzine *et al.*, 2003; Selma *et al.*, 2009). Some of the liver conjugates are then excreted as bile components and back into the intestine (enterohepatic recirculation), while deconjugated compounds are regenerated by gut microbial enzymes before being reabsorbed again (Cardona *et al.*, 2013). The unabsorbed metabolites are eliminated via faeces. All these conjugation mechanisms are highly efficient, and free aglycones are generally absent or present in low concentrations in plasma after nutritional doses (Marín *et al.*, 2015).

In opposition, few studies have been performed regarding the absorption, tissue distribution, metabolism and excretion of flavone *C*-glycosides (Sheng *et al.*, 2014; Xue *et al.*, 2014) in spite of the importance of the biodisposition of flavone *C*-glycosides after oral administration, which determines their potential benefit in pharmacotherapy or nutrition. In addition to the structural and physico-chemical attributes of the parent compounds, their pharmacokinetics is essential because determines their biological effects. However, the current understanding of absorption and biodisposition is limited to a small number of dietary flavonoids and few studies referred to the metabolism of flavone *C*-glycosides (Ma *et al.*, 2010; Zhang *et al.*, 2010, 2007). A single oral dose administration of a *Crataegus* crude extract evidenced that only the intact forms of vitexin *O*-glucoside and *O*-

rhamnoside were detected in plasma, tissues, urine and bile and that deconjugation of *C*-glycosyl flavone was not a prerequisite for its absorption in rats (Ma *et al.*, 2010). Similarly, when orally administered, puerarin, another *C*-glycosyl flavone, was also rapidly absorbed from the intestine without being previously metabolized (Prasain *et al.*, 2004). High recovery of administered vitexin 4''-*O*-glucoside and 2''-*O*-rhamnoside in faeces indicated that the efficient absorption of these molecules from the gastro-intestinal tract of rats was low. In fact, it was reported that vitexin 2''-*O*-rhamnoside has limited gastrointestinal absorption with an oral bioavailability of only 3.57% (Liang *et al.*, 2007; Zhang *et al.*, 2010). A recent work investigated the pharmacokinetic and excretion profile of *Swertia pseudochinensis* extract after its oral administration to rats and determined three secoiridoid glycosides and three flavonoid glycosides in plasma, bile, urine and faeces (Sheng *et al.*, 2014). This study also found that, although both groups of components rapidly achieved the maximum peak concentration in plasma (C_{max}) with a corresponding time to reach C_{max} (T_{max}) between 10 and 40 min, the bioavailability of the secoiridoid glycosides was significantly higher than that of the flavonoid glycosides and the elimination half-life ($T_{1/2}$) ranged from 58.4 to 263.0 min. These results demonstrated that both the absorption and elimination processes of all analytes were fast, and mainly by urine excretion.

The objective of the current study was to investigate, for the first time, the pharmacokinetic profile of phenolic acids and flavonoids in rats after oral administration of a single dose of *C. citratus* infusion which has very recently revealed anti-inflammatory activity *in vivo* (Garcia *et al.*, 2015).

2. Materials and Methods

2.1 Chemicals and drugs

Isovitexin (ISV), isoorientin (ISO), luteolin (LUT), luteolin 7-*O*-glucuronide (L7G), chrysoeriol (CHR) and diosmetin (DIO) were purchased from Extrasynthese® (Lyon, France). Caffeic acid (CAF) and *p*-coumaric acid (COU) were acquired from Sigma-Aldrich® (St. Louis, MO, USA). Rutin (RUT), sodium chloride, potassium chloride, disodium phosphate, monopotassium phosphate,

formic acid, acetone, *n*-hexane and methanol (HPLC gradient grade) were purchased from Merck® (Darmstadt, Germany). Ultra-pure water (HPLC grade >18 MΩ; homemade) was prepared by means of a MilliQ water apparatus from Millipore® (Milford, MA, USA). Isoflurane (Isoflo®) was acquired to Siloal, S.A.

2.2 Plant material and extraction

Dry leaves of *Cymbopogon citratus* were acquired from Ervital® (Mezio, Castro Daire, Portugal). *Cymbopogon citratus* was bred in the region of Mezio, Castro Daire (Portugal) and 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 (Life Sciences Department, University of Coimbra, Portugal).

A lipid- and essential oil-free infusion was prepared as previously described (Figueirinha *et al.*, 2008). The infusion was obtained by adding 150 mL of boiling water to 5 g of powdered plant material, the mixture being kept hot and left to stand for 15 min. The extract was then washed with *n*-hexane (1:1) three times to eliminate the lipophilic compounds and filtered under vacuum, concentrated in a rotavapor and freeze-dried. This freeze-dried extract (CcI) was kept at -40ºC, until further use.

2.3 *In vivo* pharmacokinetic studies

All the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for animal experiments and approved by the Portuguese Veterinary General Division.

Adult male Wistar rats (330-380 g) were obtained from Charles River (Barcelona, Spain), maintained under controlled environmental conditions (temperature 20 ± 2ºC; relative humidity 55 ± 5%; 12 h light/dark cycle) and receiving a standard rodent diet (4RF21, Mucedola®, Italy) during almost all experimental procedures and tap water *ad libitum*. At the night before CcI administration, animals were anesthetized with isoflurane and their lateral tail vein was cannulated by inserting the Introcan® Certo IV indwelling cannula (22 G; 0.9 x 2.5 mm) for serial blood sampling. The rats fully recovered from anesthesia

overnight, and were fasted for 12–14h before CcI administration with free access to water. An additional fasting period (4 h post-dose) was considered to avoid the effect of food on the oral bioavailability. A single-dose (68.24 mg/kg) of a CcI aqueous solution was administered by oral gavage to male Wistar rats (4 mL/kg of body weight). The dose of CcI was calculated based on the traditionally used doses, and taking into account the Food and Drug Administration (FDA) Guidance for Industry on conversion of animal doses to human equivalent doses according to body surface area (US DHHS, FDA, 2005).

In the plasma pharmacokinetic study, six rats (n = 6) were treated with the single-dose of CcI extract aqueous solution (68.24 mg/kg, p.o.) and multiple serial blood samples (approximately 0.3 mL) were collected through the cannula into heparinized tubes at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h post-dosing. Blood sampling was conducted in conscious and freely moving rats, which were appropriately restrained only at the moment of blood collection. Blood samples were immediately centrifuged at 3000 g for 10 min (4ºC) to separate the plasma, which was stored at -80ºC until analysis.

In the tissue pharmacokinetic studies, liver and kidneys were harvested, after decapitation and exsanguination under anesthesia, at 1, 2, 4, 8 and 24 h following CcI administration at the same dose of plasma pharmacokinetic study (n = 2, per time). The organs were excised and stored at -80ºC; the tissues were weighed and homogenized in phosphate buffer saline (PBS) pH 7.4 (4 mL per gram of tissue) before analysis.

2.4 Samples analysis

Concentrations of analytes in plasma and tissues (liver and kidneys) were determined by using a solid-phase extraction procedure followed by a reversed-phase high performance liquid chromatography (HPLC) analysis, according to a previously validated method with slight modifications (Costa *et al.*, 2015b).

Briefly, 20 µL of a 50% methanolic solution of internal standard (RUT) (100 µg/mL) was added to an aliquot of rat plasma sample (100 µL) or tissue homogenate supernatant sample (1 mL) and mixed with 400 µL of PBS buffer.

Afterwards, the pre-treated sample was subjected to a solid-phase extraction (SPE) on the Oasis® HLB (30 mg, 1 mL) cartridge (Waters®, Milford, MA, USA), which was previously conditioned with 1 mL of 0.5% formic acid in methanol followed by 1 mL of 0.5% aqueous formic acid. The loaded cartridge was subsequently submitted to −60 kPa and washed four times with 1 mL of 0.5% aqueous formic acid. After drying the sorbent under vacuum for 5 min, analytes were eluted with 1 mL of methanol-acetone (50:50, v/v) using a gentle vacuum. The eluate was evaporated to dryness at 40ºC in a vacuum oven and reconstituted with 150 µL of water–methanol (50:50, v/v) by vortexing and ultrasonication. At last, 100 µL of the final mixture were injected into the chromatographic system.

HPLC analyses were performed according to Costa *et al.* (Costa *et al.*, 2015b), by using a chromatograph equipped with a photo diode-array detector (PDA) (Gilson® Electronics SA, Villiers le Bel, France). The studies were carried out on a Spherisorb S5 ODS-2 column (250 x 4.6 mm i.d.; particle size, 5 μm; Waters® Corp., Milford, MA, USA) at 25ºC and a Nucleosil C_{18} guard cartridge (30 x 4 mm i.d.; particle size, 5 μm; Macherey-Nagel, Düren, Germany). A mobile phase of 5% (v/v) aqueous formic acid (A) and methanol (B) was used with a discontinuous gradient: 5–15% B (0–10 min.), 15–30% B (10–15 min.), 30–35% B (15–25 min.), 35–50% B (25–35 min.) and 50–80% B (35–40 min.), followed by an isocratic elution for 20 min., at a flow rate of 1 mL/min. An injection volume of 100 μL was used for all standards and samples. Chromatographic profiles were acquired in the wavelength range 200–600 nm, and recorded at 280 and 320 nm. The quantification of each compound was achieve through the absorption at 320 nm. Data treatment was carried out with Unipoint® 2.10 software (Gilson®). The main partial validation parameters of the analytical method employed were in agreement with the international guidelines (EMA, 2011; FDA, 2013) and are summarized in **Table II.12**.

HPLC-PDA-ESI/MS^n analysis, based on a previously optimized method (Tavares *et al.*, 2015), was performed in a Surveyor® liquid chromatograph hyphenated to a photo-diode-array detector (PDA) (Surveyor®) and a Finnigan® LCQ mass spectrometer (San Jose, CA, USA) equipped with an API-ES ionization

chamber. Separation was performed on a Spherisorb® ODS-2 C18 reverse phase column, 150 x 2.1 mm i.d. and particle size of 3 μm (Waters® Corporation, Milford, Massachusetts, USA) and a Spherisorb® ODS-2 guard cartridge C_{18} (10 x 4.6 mm i.d. and particle size of 5 μm; Waters® Corporation, Milford, Massachusetts, USA) at 25ºC, using 2% aqueous formic acid (A) and methanol (B) as mobile phase. The gradient profile used was 5-15% B (0-10 min), 15-30% B (10-15 min), 30-35% B (15-25 min), 35-50% B (25-35 min), 50-80% B (35-40 min) and 80% B (40-60 min), isocratically, at a flow rate of 0.2 mL/min. The first detection was done with the PDA detector in a wavelength range of 200-600 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 MS^1 (*m/z* 160-1300), MS^2 of the most abundant ion in MS^1, and MS^3 of the most abundant ion in MS^2. Source voltage was 4.5 kV and capillary voltage and temperature were -10 V and 250ºC, respectively. Nitrogen was used as sheath gas at flow rate of 20 arbitrary units. The normalized energy of collision was 45%, using helium as collision gas.

Table II.12. Validation parameters of the HPLC method employed for the quantification of caffeic acid (CAF), *p*-coumaric acid (COU), isovitexin (ISV), isoorientin (ISO), luteolin (LUT), luteolin 7-*O*-glucuronide (L7G), chrysoeriol (CHR) and diosmetin (DIO) in plasma, liver and kidney homogenate supernatants (n = 3) (cont.).

Analyte	Validation parameters	Plasma	Liver	Kidney
CAF	Calibration range	1-30 µg/mL	0.4-12 µg/g	0.4-12 µg/g
	Coefficient of determination (r^2)	0.9944	0.9872	0.9980
	LLOQ	1 µg/mL	0.4 µg/g	0.4 µg/g
	Precision (%CV)[a]	≤ 3.13	≤ 14.84	≤ 2.30
	Accuracy (%Bias)[a]	-7.21 – 14.61	-5.76 – 8.42	-4.32 – 5.07
	Recovery (%)	98.2 – 101.0	74.3 – 91.9	78.6 – 92.5
COU	Calibration range	1-30 µg/mL	0.4-12 µg/g	0.4-12 µg/g
	Coefficient of determination (r^2)	0.9993	0.9816	0.9960
	LLOQ	1 µg/mL	0.4 µg/g	0.4 µg/g
	Precision (%CV)[a]	≤ 5.90	≤ 16.70	≤ 4.65
	Accuracy (%Bias)[a]	-3.19 – 19.89	-2.81 – 12.71	-9.36 – 7.18
	Recovery (%)	88.7 – 89.2	75.0 – 91.3	74.9 – 90.5

Table II.12. Validation parameters of the HPLC method employed for the quantification of caffeic acid (CAF), *p*-coumaric acid (COU), isovitexin (ISV), isoorientin (ISO), luteolin (LUT), luteolin 7-*O*-glucuronide (L7G), chrysoeriol (CHR) and diosmetin (DIO) in plasma, liver and kidney homogenate supernatants (n = 3).

Analyte	Validation parameters	Plasma	Liver	Kidney
ISV	Calibration range	2-60 μg/mL	0.8-24 μg/g	0.8-24 μg/g
	Coefficient of determination (r^2)	0.9996	0.9858	0.9864
	LLOQ	2 μg/mL	0.8 μg/g	0.8 μg/g
	Precision (%CV)[a]	≤ 5.78	≤ 15.96	≤ 14.55
	Accuracy (%Bias)[a]	-2.58 – 1.67	-10.39 – 9.62	-18.90 – 8.45
	Recovery (%)	90.6 – 93.5	72.9 – 99.4	80.5 – 95.6
ISO	Calibration range	2-60 μg/mL	0.8-24 μg/g	0.8-24 μg/g
	Coefficient of determination (r^2)	0.9988	0.9872	0.9979
	LLOQ	2 μg/mL	0.8 μg/g	0.8 μg/g
	Precision (%CV)[a]	≤ 4.69	≤ 14.84	≤ 8.83
	Accuracy (%Bias)[a]	-18.28 – 8.99	-5.76 – 8.42	-3.84 – 5.24
	Recovery (%)	89.4 – 91.1	73.5 – 98.6	76.0 – 86.2
LUT	Calibration range	2-60 μg/mL	0.8-24 μg/g	0.8-24 μg/g
	Coefficient of determination (r^2)	0.9969	0.9910	0.9979
	LLOQ	2 μg/mL	0.8 μg/g	0.8 μg/g
	Precision (%CV)[a]	≤ 5.48	≤ 20.34	≤ 8.83
	Accuracy (%Bias)[a]	-6.42 – 8.01	-4.87 – 11.02	-3.84 – 5.24
	Recovery (%)	81.2 – 93.2	72.3 – 86.2	70.5 – 82.9
L7G	Calibration range	2-60 μg/mL	0.8-24 μg/g	0.8-24 μg/g
	Coefficient of determination (r^2)	0.9969	0.9910	0.9979
	LLOQ	2 μg/mL	0.8 μg/g	0.8 μg/g
	Precision (%CV)[a]	≤ 5.48	≤ 20.34	≤ 8.83
	Accuracy (%Bias)[a]	-6.42 – 8.02	-4.79 – 11.07	-3.84 – 5.24
	Recovery (%)	82.7 – 93.2	78.9 – 89.6	77.9 – 87.6
CHR	Calibration range	2-60 μg/mL	0.8-24 μg/g	0.8-24 μg/g
	Coefficient of determination (r^2)	0.9969	0.9910	0.9979
	LLOQ	2 μg/mL	0.8 μg/g	0.8 μg/g
	Precision (%CV)[a]	≤ 5.48	≤ 20.34	≤ 8.83
	Accuracy (%Bias)[a]	-6.43 – 8.01	-4.73 – 11.08	-3.84 – 5.23
	Recovery (%)	82.9 – 92.6	80.2 – 86.2	79.2 – 83.5
DIO	Calibration range	2-60 μg/mL	0.8-24 μg/g	0.8-24 μg/g
	Coefficient of determination (r^2)	0.9969	0.9910	0.9979
	LLOQ	2 μg/mL	0.8μg/g	0.8 μg/g
	Precision (%CV)[a]	≤ 5.48	≤ 20.34	≤ 8.83
	Accuracy (%Bias)[a]	-6.43 – 8.00	-4.78 – 11.06	-3.84 – 5.24
	Recovery (%)	84.9 – 94.0	79.2 – 84.3	80.3 – 82.9

[a]Inter-day values, n = 3; Bias, deviation from nominal value; CV, coefficient of variation: LLOQ, lower limit of quantification; NC, not calculated.

2.5 Pharmacokinetic analysis

The plasma concentration *versus* time data for each analyte obtained from each individual rat was submitted to a noncompartmental pharmacokinetic analysis using the WinNonlin® version 5.2. (Pharsight Co, Mountain View, CA, USA).

The C_{max} in plasma and tissues of each analyte and the corresponding T_{max} were directly derived from the experimental data obtained. The remaining pharmacokinetic parameters evaluated included: the area under the drug concentration time-curve (AUC) from time zero to the last sampling time at which quantifiable drug concentrations were determined ($AUC_{0\rightarrow t}$), calculated by the linear trapezoidal rule; the AUC from time zero to infinite ($AUC_{0\rightarrow inf}$) that was calculated from $AUC_t + (C_{last}/k_{el})$, where C_{last} is the last quantifiable concentration and k_{el} is the apparent elimination rate constant estimated by log-linear regression of the terminal segment of the concentration–time profile; the apparent terminal elimination half-life ($T_{1/2}$) and the mean residence time (MRT).

3. Results and Discussion

The developed method was validated for eight different compounds: CAF, COU, ISV, ISO, LUT, L7G, CHR and DIO. L7G, L3'S, LUT, CHR and DIO were identified by HPLC-PDA-ESI/MS^n. All compounds presented UV spectra characteristic of luteolin derivatives, with λ_{max} at 251-255 and 344-349 nm. L7G ($[M - H]^-$ at *m/z* 461) presented MS^2 fragments at *m/z* 357, 327, 285 and 115, suggesting the presence of luteolin 7-*O*-glucuronide (Yin *et al.*, 2013). L3'S ($[M - H]^-$ at *m/z* 365) showed a MS^2 fragmentation at *m/z* 285 and 257, associated with the chemical structure of luteolin *O*-sulfate. LUT ($[M - H]^-$ at m/z 285) showed the typical product-ions from the fragmentation of luteolin aglycone: *m/z* at 257, 241, 199, 175, 151 and 133 (Roriz *et al.*, 2014). CHR and DIO exhibited the same parent-ion ($[M - H]^-$ at *m/z* 299), and very similar fragmentation patterns: *m/z* at 284, 255, 151, 132, 107 and 284, 255, 179, 151, 107, respectively; indicating the presence of *O*-methylated luteolin derivatives, chrysoeriol and diosmetin, respectively (Brito *et al.*, 2014; Radwan and Hamdy, 2006). Since the

method was not validated to L3'S due to the unavailability of standard, this analyte was quantified expressing the results in equivalents of L7G. In fact, this is an approach often employed by other authors (Granica *et al.*, 2015; Kiselova *et al.*, 2006).

Globally, the present pharmacokinetic study, in plasma and tissues, revealed the presence of LUT, L7G, CHR, DIO and also L3'S (**Figure II.23**), while CAF, COU, ISV and ISO were not detected by the validated bioanalytical method, in all matrices.

Although L7G and L3'S are not present in CcI, they were the only ones quantified in rat plasma. The plasma concentration-time profiles of the analytes found and quantified in rat plasma are shown in **Figure II.24** and the corresponding estimated pharmacokinetic parameters are listed in **Table II.13**. Taking them together, it becomes evident that L7G and L3'S rapidly reached T_{max}, with maximum concentrations at 30 min after oral administrations of CcI extract. Since they are not present in the initial extract and they quickly reach the C_{max}, it can be possible to infer that the parent compounds are rapidly metabolized, probably in the gut and liver, before reaching the systemic blood flow. In fact, it is known that, at the intestinal tract, luteolin glycosides are hydrolyzed to luteolin, which is rapidly metabolized to glucuronides and sulfates by UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and/or microbiota enzymes (Davis and Brodbelt, 2008; Tomás-Barberán and Andrés-Lacueva, 2012). Moreover, luteolin is also described as being rapidly metabolized to L7G by UGTs, or to L3'S by SULTs in the liver (Tomás-Barberán and Andrés-Lacueva, 2012). Although plasma concentration-time profiles for L7G and L3´S are almost parallel (**Figure II.24**), their pharmacokinetic parameters in plasma after oral administration of *C. citratus* extract revealed to be slightly different from each other (**Table II.13**), with L7G presenting higher values of C_{max}, AUC, $T_{1/2}$ and MRT. These findings, suggest not only a higher biodisposition in plasma but also that L7G remains for a longer period of time in the organism. These data are in accordance with previous works regarding the plasma pharmacokinetics of similar compounds, such as vitexin 2''-*O*-rhamnoside, vitexin 4''-*O*-glucoside, luteolin 3'-

O-glucuronide, apigenin 7-*O*-glucuronide and acacetin 7-*O*-glucuronide (Liang *et al.*, 2007; Qiang *et al.*, 2015; Zhang *et al.*, 2010). As for the L3'S, it was already reported as being a major ISO metabolite in rats (Shi *et al.*, 2015).

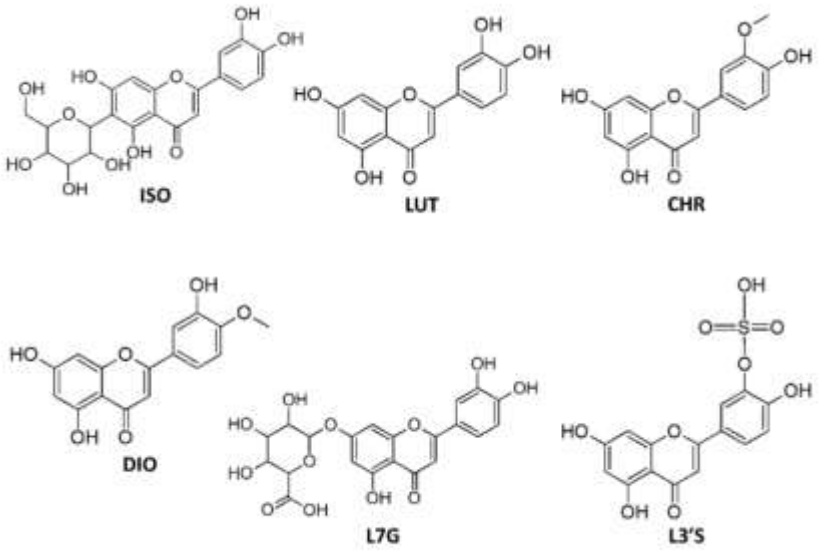

Figure II.23. Molecular structures of luteolin (LUT) and derivatives (isoorientin, ISO; chrysoeriol, CHR; diosmetin, DIO; luteolin 7-*O*-glucuronide, L7G; luteolin 3'-*O*-sulfate, L3'S).

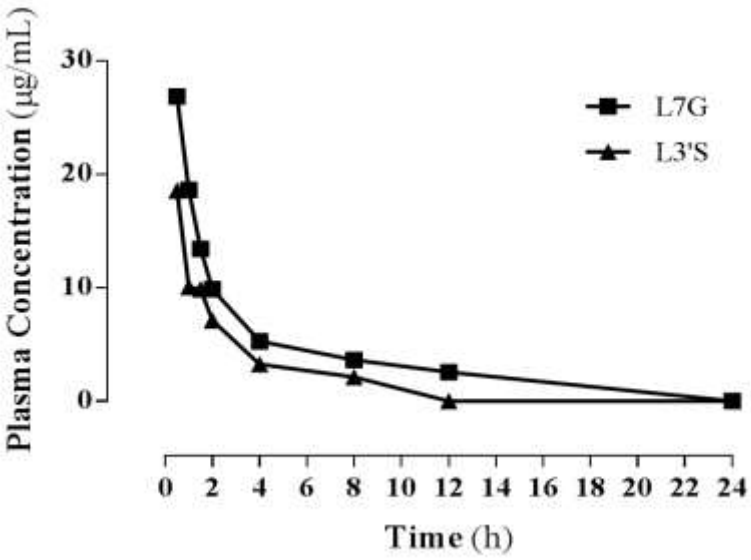

Figure II.24. Plasma concentration-time profiles of luteolin 7-*O*-glucuronide (L7G) and luteolin 3'-*O*-sulfate (L3'S) obtained after administering a single oral dose of CcI extract to rats (68.24 mg/kg). Symbols represent the mean values ± standard error of the mean of six determinations per time point (n = 6).

Table II.13. Pharmacokinetic parameters of luteolin 7-*O*-glucuronide (L7G) luteolin 3'-*O*-sulfate (L3'S), in rat plasma after oral administration of the CcI extract at 68.24 mg/kg (mean ± standard deviation, $n = 6$).

Pharmacokinetic parameters	Compounds	
	L7G	L3'S
C_{max} (μg/mL)	26.87 ± 0.89	18.57 ± 0.31
T_{max} (h)	0.50 ± 0.00	0.50 ± 0.00
$T_{1/2}$ (h)	7.61 ± 0.34	3.03 ± 0.13
$AUC_{(0\rightarrow t)}$ (h μg/mL)	77.34 ± 2.26	41.95 ± 1.11
$AUC_{(0\rightarrow \infty)}$ (h μg/mL)	105.36 ± 2.79	51.22 ± 1.37
$MRT_{(0\rightarrow \infty)}$ (h)	3.77 ± 0.04	2.57 ± 0.03

Besides L7G and L3´S, the analysis of liver tissue samples, revealed the presence of compounds undetected in plasma, namely LUT, CHR and DIO. The respective concentration-time profiles and pharmacokinetic parameters of the five analytes are displayed in **Figure II.25** and **Table II.14**, respectively. According to C_{max} and AUC values, it becomes clear that the three major metabolites found in liver tissues were L7G, CHR and L3'S, which were detected from the first hour and remained in the tissue up to at least 24h. In opposition, DIO was detected only 8 h post-dosing, exhibiting the lowest concentration of all the analytes studied, suggesting that it is a minor product of rat metabolism. Therefore, it is not expected to contribute significantly to the pharmacological effects of the CcI extract in spite of exhibiting pharmacological activity in other studies when highly bioavailable *in vivo* (Patel *et al.*, 2013).

The earlier T_{max} values found in plasma rather than in liver, suggest that these metabolites are mainly produced at the small intestine level rather than in the liver. Similarly, Courts & Williamson recently reported that flavones *C*-glycosides (e.g. vitexin, isoorientin and mangiferin) are promptly metabolized by gut microbiota and enterocytes enzymes (Courts and Williamson, 2013). This may explain the very low bioavailability of luteolin aglycone herein found.

The kidney concentration-time profile revealed the presence of the same compounds detected in plasma, L7G and L3'S (**Figure II.25**), probably due to their high concentrations in plasma. The pharmacokinetic parameters (**Table**

II.14) of these analytes are very similar, L7G presenting higher C_{max} and AUC values, but slightly lower $T_{1/2}$ and MRT values than L3'S.

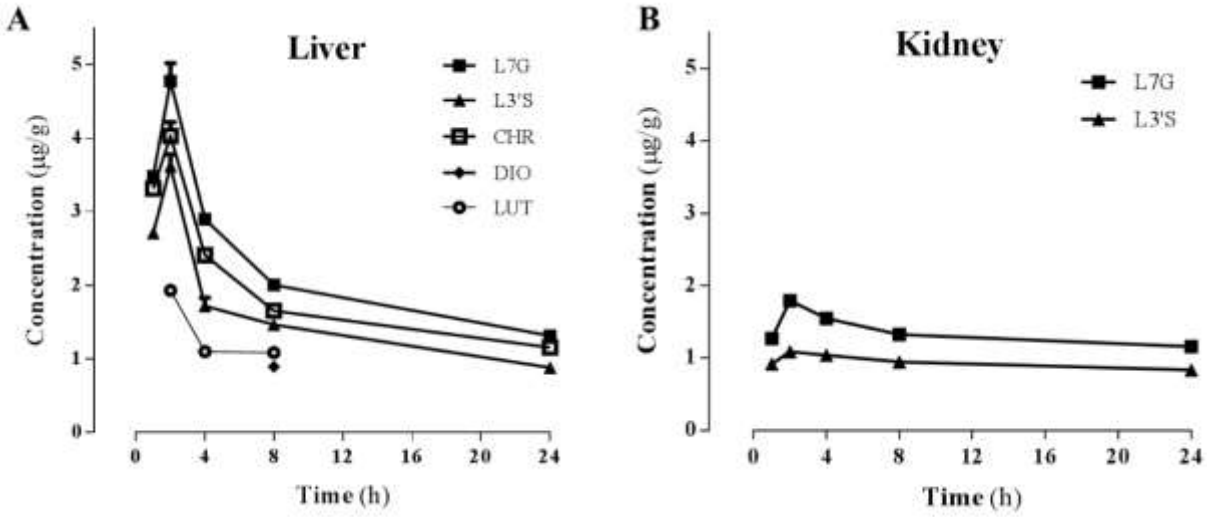

Figure II.25. Liver (**A**) and kidney (**B**) concentration-time profiles of luteolin 7-*O*-glucuronide (L7G) luteolin 3'-*O*-sulfate (L3'S), chrysoeriol (CHR), diosmetin (DIO) and luteolin (LUT) obtained after administering a single oral dose of CcI extract (68.24 mg/kg) to rats. Symbols represent the mean values ± standard error of the mean of two determinations per time point (n = 2).

Table II.14. Pharmacokinetic parameters in rat liver and kidney after p.o. administration at 68.24 mg/kg (mean ± standard deviation, n = 2).

	C_{max} (µg/g)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{(0\rightarrow t)}$ (h µg/g)	$AUC_{(0\rightarrow \infty)}$ (h µg/g)	$MRT_{(0\rightarrow \infty)}$ (h)
Liver						
L7G	4.78 ± 0.35	2.00 ± 0.00	19.40 ± 0.51	49.90 ± 1.26	86.70 ± 0.11	9.30 ± 0.10
L3'S	3.62 ± 0.23	2.00 ± 0.00	21.60 ± 5.08	35.00 ± 0.55	62.71 ± 7.74	9.21 ± 0.25
CHR	4.03 ± 0.27	2.00 ± 0.00	21.20 ± 0.89	42.40 ± 1.53	77.80 ± 4.95	6.94 ± 0.08
DIO	0.89 ± 0.04	8.00 ± 0.00	NC	NC	NC	8.00 ± 0.00
LUT	1.93 ± 0.01	2.00 ± 0.00	8.38 ± 0.39	8.37 ± 0.18	21.50 ± 1.21	4.35 ± 0.02
Kidney						
L7G	1.80 ± 0.01	2.00 ± 0.00	54.61 ± 4.38	31.14 ± 0.39	122.63 ± 9.94	11.37 ± 0.09
L3'S	1.09 ± 0.00	2.00 ± 0.00	68.88 ± 2.08	21.83 ± 0.29	104.88 ± 4.41	11.58 ± 0.05

NC, not calculated.

The pharmacokinetic analysis herein performed in plasma, liver and kidney showed that the compounds present in CcI (Costa *et al.*, 2015a; Figueirinha *et al.*,

2010, 2008; Francisco *et al.*, 2014; Orrego *et al.*, 2009) are not abundant in plasma, liver or kidneys after a 68.24 mg/kg single-dose oral administration of the extract. In fact, the metabolites, L7G and L3'S, were the most abundant in plasma, particularly L7G which presented the highest AUC values, and, therefore, they are probably the main responsible for the anti-inflammatory activity previously reported (Garcia *et al.*, 2015). Importantly it seems that, during the inflammatory process, the enzyme *β*-glucosidase overexpressed by neutrophils leads to the deglucuronidation of L7G to LUT, which, in turn, exerts the biological effect when reaching the target-tissue (Chen *et al.*, 2014; Lamy *et al.*, 2015; Shimoi, 2001; Shimoi *et al.*, 2000). Therefore, L7G and L3'S seem to be the main active circulating forms of the aglycone luteolin.

The work herein exposed revealed, for the first time, how luteolin *C*-glycosides are metabolized *in vivo* after CcI administration to rats, and which are the main metabolites probably responsible for the CcI pharmacological effects previously observed *in vivo* (Garcia *et al.*, 2015).

4. Conclusions

In conclusion, to the best of our knowledge, this work is the first report documenting the quantification of LUT, L7G, L3'S, CHR and DIO in rat plasma, liver and/or kidney, after the oral administration of a single dose of *C. citratus* infusion. This work has successfully investigated the pharmacokinetics of CcI in rats, and could also be applied in future studies of lemongrass preparations. The pharmacokinetics of the two major luteolin conjugates *in vivo* (L7G and L3'S) herein obtained support the pharmacological effects of *C. citratus in vivo*.

GENERAL DISCUSSION and CONCLUSIONS

CHAPTER III

Cymbopogon citratus (lemongrass) is an herb commonly used in traditional medicine for gastrointestinal disorders, namely gastritis, diarrhea, stomach problems, abdominal pain, digestive ailments, and for diseases related with inflammation, such as fever, arthritis, rheumatism, muscular pain, colds, cough (**Chapter I**). These several traditional uses are associated to lemongrass leaves and may be linked to the antioxidant, gastroprotective and/or anti-inflammatory properties, which were intended to be validated in this dissertation. In order to assess the phytochemicals that may be responsible for the referred traditional uses, in the past years, great attention has been payed to the phenolic compounds present in this plant and to their anti-inflammatory effects (Figueirinha *et al.*, 2010, 2008; Francisco *et al.*, 2011). The anti-inflammatory activity verification of phenolic compounds enriched fractions, through *in vitro* assays, suggests that these phytochemicals may be suitable for extracts standardization, which, in turn, may be used in *C. citratus* derivated anti-inflammatory products. Therefore a validated method is crucial to obtain standardized extracts or products, in order to guarantee the proper quality for human consumption.

A. Development and validation of a simple and sensitive RP-HPLC method for quantitation of flavonoids in Cymbopogon citratus infusion

In this work, a simple and efficient RP-HPLC-PDA method was successfully validated (**section II.A**) for simultaneous identification and quantification of phenolic compounds, namely phenolic acids and flavonoids, for the first time, in three different extracts: infusion and 50% aqueous ethanol and ethanol macerates. The developed HPLC method was validated according to ICH and FDA guidelines. The validated method is simple, precise and accurate, presenting acceptable LOD and LOQ values. It may also be applied for phenolic compounds quantification in other polar extracts as well as for quality control of herbal formulations, with promising use in pharmaceutical and food supplement industries.

B. Influence of harvest time and material quality on phenolic content and antioxidant activity of Cymbopogon citratus infusion

In the case of *C. citratus*, this crop is harvested along a wide period of the year (from the beginning of spring till the end of summer). Additionally, it is known that phenolic compounds vary with sunlight exposure and climatic conditions (Booij-James *et al.*, 2000; Cohen *et al.*, 2008; Spayd *et al.*, 2002). So, the amount of these compounds in the plant leaves may suffer significant variations, leading to chemically and, therefore, therapeutically different products, the phenolic compounds characterization and quantification revealing to be of extraordinary importance. In order to address that issue, a work to study the influence of harvest time, but also the plant quality on lemongrass phenolic composition and antioxidant power was carried out (**section II.B**). Our results indicated that the content on polyphenols and the antioxidant capacity of *C. citratus* infusion is strongly related with the quality of the plant at the moment it is harvested. The total phenols assay showed a substantial loss from August to September, suggesting that the biosynthesis of these compounds is affected by the sunlight. Moreover, each group of phenolic compounds is affected by the harvest time. Therefore, it is not indifferent to extract the phenolic compounds from this plant with different quality grades or harvested on different months of the year. It was possible to find out the best month to harvest the plant to get the most of each phenolic group: April and June for hydroxycinnamic acids; June and September for flavonoids; June, July and August for tannins. Regarding the influence of plant quality on the composition of lemongrass extract, it is clear the correlation between these two parameters. Regardless the group of phenolic compound addressed, its content was always inversely proportional to the degree of leaves ageing, and therefore, to the time of sunlight and/or UV light exposition. This work also shed lights on how the harvest time and the oxidation state of the plant leaves affected the polyphenolic profile and antioxidant activity. Among the different groups of phenolic compounds, both phenolic acids and tannins seem to be the most decisive in the overall antioxidant activity of the extract. In what concerns the quality of the harvested material and its antioxidant capacity, the relationship could not be more explicit. For all tested oxidant species, the high-

quality samples exhibited the best antioxidant results, and samples collected at the same month, but with low-quality presented the poorest potential.

To sum up, both harvest date and plant quality are key criteria that have to be taken into account when selecting the material for human consuming, in order to receive benefits that have been allocated to phenolic compounds, namely to those who are present in this plant. In addition, these results indicate that the plant must be protected from light, also during the post-harvest stage, namely during drying, chopping and packing processes.

These results taken together with those of section **II.A** revealed that the extraction method and essentially the extraction solvent have a much greater influence on flavonoid amount than the harvest date. On the other hand, the harvest date is more determinant for the phenolic acids amount than the solvent used. To further investigate the relationship between the extraction solvent and the quality and quantity of phenolic compounds extracted, a complementary study was carried out (see **Attach 2**). This study confirmed how flavonoids are greatly influenced by the extraction solvent, its contents being greatly increased when an ethanol percentage of 80% or more is used.

The results showed that also tannins are remarkably influenced. In the case of this group of polyphenols, water or a hydroalcoholic mixture up to 60% ethanol should be used. Higher percentages of ethanol severely affect the tannins extraction yield. Additionally, the extraction solvent influence on the antioxidant power was also investigated. The results (see **Attach 3**) showed that the extraction process also has an important role on the antioxidant capacity. In the case of DPPH radical, the best solvents are the 50% and 60% aqueous ethanol, while 40% aqueous ethanol extraction leads to the poorest scavenging activity. For FRAP, 50% ethanolic extract proved to be the one with the highest activity, being 100% ethanolic extract the weakest one. In ABTS (pH=7), there were not significant differences among the studied extraction solvents. On the other hand, 50% aqueous ethanol demonstrated once again to be the one solvent to extract the greatest amount of antioxidant compounds against ABTS radical (pH=4), while *C. citratus* infusion and absolute ethanol extract were the less effective. These results

corroborate the previous ones, demonstrating that the solvents which better extract tannins lead to extracts with greater antioxidant activities against the tested oxidant species. Another complementary study was performed to observe the antioxidant effect of each enriched fraction of phenolic acids, flavonoids and tannins (**Attach 4**). In this study, it was observed that both tannins and flavonoids have a greater antioxidant capacity than the *C. citratus* infusion, for all tested radicals. Moreover, the tannin fraction is the strongest one in what concerns antioxidant activity, even stronger than Trolox®, with the exception of ABTS at pH=4. In this case, the phenolic acids fraction surpasses the tannins. This fact may be associated with the pK of the referred compounds, which favors the performance of phenolic acids at acidic environments, and the tannins at higher pHs (Friedman and Ju, 2000). This certainly has clinical implications, namely if the compounds are administrated *per os*. Hence, tannins and phenolic acids are tightly associated with the overall activity of lemongrass extracts. On the other hand, the tannin fraction is the one showing the best activity in FRAP assay. This fact may be explained by the known property of metal ions complexation attributed to tannins (Schmidt *et al.*, 2013).

C. Flavan hetero-dimers in the Cymbopogon citratus infusion: tannin fraction obtaining, evaluation of its contribution to the antioxidant activity and structural elucidation

On the other hand, tannins and flavonoids have been reported to possess anti-inflammatory properties *in vitro* (Figueirinha *et al.*, 2010; Francisco *et al.*, 2014, 2011). Since the chemical characterization of flavonoids has already been disclosed (Figueirinha *et al.*, 2008; Francisco *et al.*, 2014), the necessity to characterize the tannins structure was evident.

A study by HPLC-PDA-MS^n identified, for the first time in *C. citratus*, hetero-dimeric flavan structures (**section II.C**). These flavonoid oligomers consist of apigeniflavan or luteoliflavan units linked to a flavanone, either naringenin or eriodictyol, and occur as aglycone and glycosylated forms. The detected proanthocyanidin hetero-dimers, along with some common

procyanidin dimers, constitute the main compounds in the tannin fraction of lemongrass. The findings of this study support the antioxidant potential of this plant and emphasize the contribution of the tannin fraction to this activity. Furthermore, tannin-rich fractions from *C. citratus* leaves have already been associated with antioxidant and anti-inflammatory properties (Figueirinha *et al.*, 2010, 2008; Francisco *et al.*, 2011) and the tannins similar to those present in *C. citratus* have also been suggested as anti-inflammatory and antioxidant (Bors *et al.*, 2000; Riedl and Hagerman, 2001), which may lead to a structure-activity relationship based on this biological effect and the tannin structure disclosed here for the first time.

D. Gastroprotective effect of Cymbopogon citratus infusion on acute ethanol-induced gastric lesions in rats

Oxidative and inflammatory processes episodes may be the root of gastric disorders (Mario *et al.*, 2014) so that in this work the gastroprotective effect of a *C. citratus* infusion, the most commonly application to this extract, was studied through an *in vivo* model.

This extract, administered before and after ethanol-induced gastric lesions (**section II.D**), significantly reduced the incidence and severity of gastric lesions and, consequently, the ulcer index. These data suggest a gastroprotective activity of *C. citratus* infusion and corroborate the traditional medicinal use of this plant to ameliorate gastritis and/or peptic ulcers symptoms, and that the antioxidant properties of lemongrass phenolic compounds could be partially responsible for the observed gastroprotective effects, as has been verified to other polyphenols (La Casa *et al.*, 2000). Additionally, the gastroprotective effect has also been associated with anti-inflammatory mechanisms (Maity *et al.*, 2009). So, both antioxidant and anti-inflammatory properties of lemongrass polyphenols may contribute to the overall gastroprotective activity of this plant.

With the purpose of further investigate the anti-inflammatory potential of *C. citratus* infusion and/or its phenolic compounds two different approaches of administration routes were taken into account: oral and topical.

E. In vivo evaluation of anti-inflammatory and analgesic activities of flavonoid and tannin fractions from Cymbopogon citratus infusion

The first *in vivo* anti-inflammatory validation of *C. citratus* after an oral administration (**section II.E**) was performed with the carrageenan-induced paw oedema and pointed the traditional use of *C. citratus* infusion (CcI) as an anti-inflammatory agent. The observed effect on oedema inhibition for an administration of 68.24 mg/kg was 82.30%, value very similar to that obtained by 10 mg/kg of the reference NSAID used -diclofenac (84.00%), flavonoid- (7.42 mg/kg) and tannin-rich (5.96 mg/kg) fractions contributing significantly to anti-inflammatory activity (59.0 and 61.0%, respectively). On the other hand, our results also showed that effective CcI concentrations induced no hepatotoxicity in rats and, therefore, the administration of CcI may be considered safe under the conditions used. These results suggest that lemongrass infusion and its polyphenols, namely the flavonoids and tannins, may be used to obtain a new anti-inflammatory medicine, effective in the treatment of inflammatory-related pathologies.

F. Polyphenols from Cymbopogon citratus leaves as topical anti-inflammatory agents

The topical anti-inflammatory activity of CcI was also addressed *in vivo* (**section II.F**). Firstly, the topical formula was optimized in order to obtain the most efficient anti-inflammatory effect *in vivo*. This feature was monitored using the carrageenan-induced paw oedema model in rats (**Attach 5**). The first formulation tested was a hydrogel which led to a 12% reduction in the oedema volume. Afterwards, an oil/water emulsion was experimented, revealing a 31% reduction. Hence, it was possible to conclude that, in this case, an oil/water emulsion is more effective in delivering the active compounds to the site of action than the hydrogel. Additionally, one or two permeability enhancers were added to the emulsion formula and the anti-inflammatory effect increased 10% and 16%, respectively. Therefore, the formula of choice was the last one tested: stearic acid,

triethanolamine, glycerol, water, propylene glycol, oleic acid, which was used in further investigations.

With the data obtained from the Franz cells releasing assay, it was possible to presume that the topical anti-inflammatory effect was due, at least in part, to the most abundant compounds detected in the receptor medium, namely luteolin 7-*O*-neohesperidoside, cassiaoccidentalin B, carlinoside and luteolin 7-*O*-glucoside. Curiously, a study performed with flavonoids glycosides reported the topical anti-inflammatory effect in rats (Antunes-Ricardo *et al.*, 2015). These data corroborate our results, which may suggest that CcI polyphenols can exert their effect *in loco*, without the necessity of being firstly metabolized by the enterocytes or hepatocytes.

Further investigations were applied to test separately the topical anti-inflammatory activities of the tannins (CcT) and flavonoids (CcF) fractions in the *in vivo* model, using the same formulation (**Attach 6**). Formulations containing 0.3% of CcT and 0.6% of CcF (concentrations calculated taken into consideration the yields of these phenolic fractions obtained from CcI) diminished the oedema volume by 47% and 43%, respectively. Regarding these results, it is possible to conclude that CcT has a greater power to reduce the inflammatory oedema than CcF. Moreover, for a CcT+CcF formulation (0.34% and 0.66%, respectively) it was verified an higher anti-inflammatory effect (59%), that is more approached to the positive control (1% diclofenac formulation), 65.9%. In light of this study, we may suggest that tannins and flavonoids of the *C. citratus* possess a synergistic mechanism of action. Anti-inflammatory synergistic effects have already been described among various flavonoids and tannins (Mythilypriya *et al.*, 2008).

In conclusion, CcI flavonoids and tannins could be active constituents to integrate in a new topical anti-inflammatory agent, effective for the treatment of skin inflammatory-related pathologies.

G. Pharmacokinetic profile of Cymbopogon citratus polyphenols in rats

Through **section II.G**, the pharmacokinetics of *C. citratus* flavonoids was studied in order to better understand the biodisposition of these compounds

present in the extract. The pharmacokinetic analysis performed in plasma, liver and kidney showed that the compounds present in CcI were not detected *in vivo* after a single-dose oral administration. In fact, the metabolites, luteolin 7-*O*-glucuronide, luteolin 3'-*O*-sulfate, luteolin, chrysoeriol and diosmetin were the most abundant in plasma, particularly luteolin 7-*O*-glucuronide and luteolin 3'-*O*-sulfate, which presented the highest bioavailability, and, consequently, they are probably the main responsible for the anti-inflammatory activity previously reported, after an oral administration. Therefore, luteolin 7-*O*-glucuronide and luteolin 3'-*O*-sulfate seems to be the main active circulating forms of the aglycone luteolin.

The work herein exposed revealed, for the first time, how luteolin glycosides are metabolized *in vivo* after CcI administration to rats, and that glucuronides and sulfates are probably the ones responsible for the *in vivo* pharmacological effects attributed to flavonoids.

To conclude, all the objectives of this thesis were fulfilled and an important piece of knowledge was added about the phytochemical polyphenols and its anti-inflammatory properties, as well as those of *C. citratus* infusion. Furthermore, for the first time, the anti-inflammatory effect, after oral and topical administration, and the pharmacokinetic profile of the compounds with therapeutic interest have been described *in vivo*.

FUTURE PRESPECTIVES

CHAPTER IV

In the future, further investigation must be undertaken in order to improve the chemical characterization of this plant, namely in what concerns its tannins. Therefore, it is appropriate to isolate and characterize each molecule, using more powerful techniques such as ^{1}H and ^{13}C NMR.

The research undertaken to evaluate the influence of the harvest time and plant quality should be extended to the anti-inflammatory activity, since this property is tightly dependent on the quality and quantity of the phenolic compounds present in lemongrass. Besides, it would be of great importance to study the degradation profile of phenolic compounds in lemongrass leaves, during the post-harvest processes (drying, chopping and packing), in different light, temperature and humidity conditions.

Also further *in vitro* and *in vivo* evaluations are required to elucidate the potential existence of synergistic behaviors.

In addition, the pharmacokinetics approach must be deepened to flavonoids and tannins fractions by studying the profile of each fraction individually and in combination. Only then, the bioavailability and the pharmacodynamic interactions between phenolic compounds and target-tissues may be fully comprehended.

Subsequently, studies in humans are absolutely crucial to better understand the biological effect of this plant in the body and thus be able to make the best of it.

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CHAPTER V

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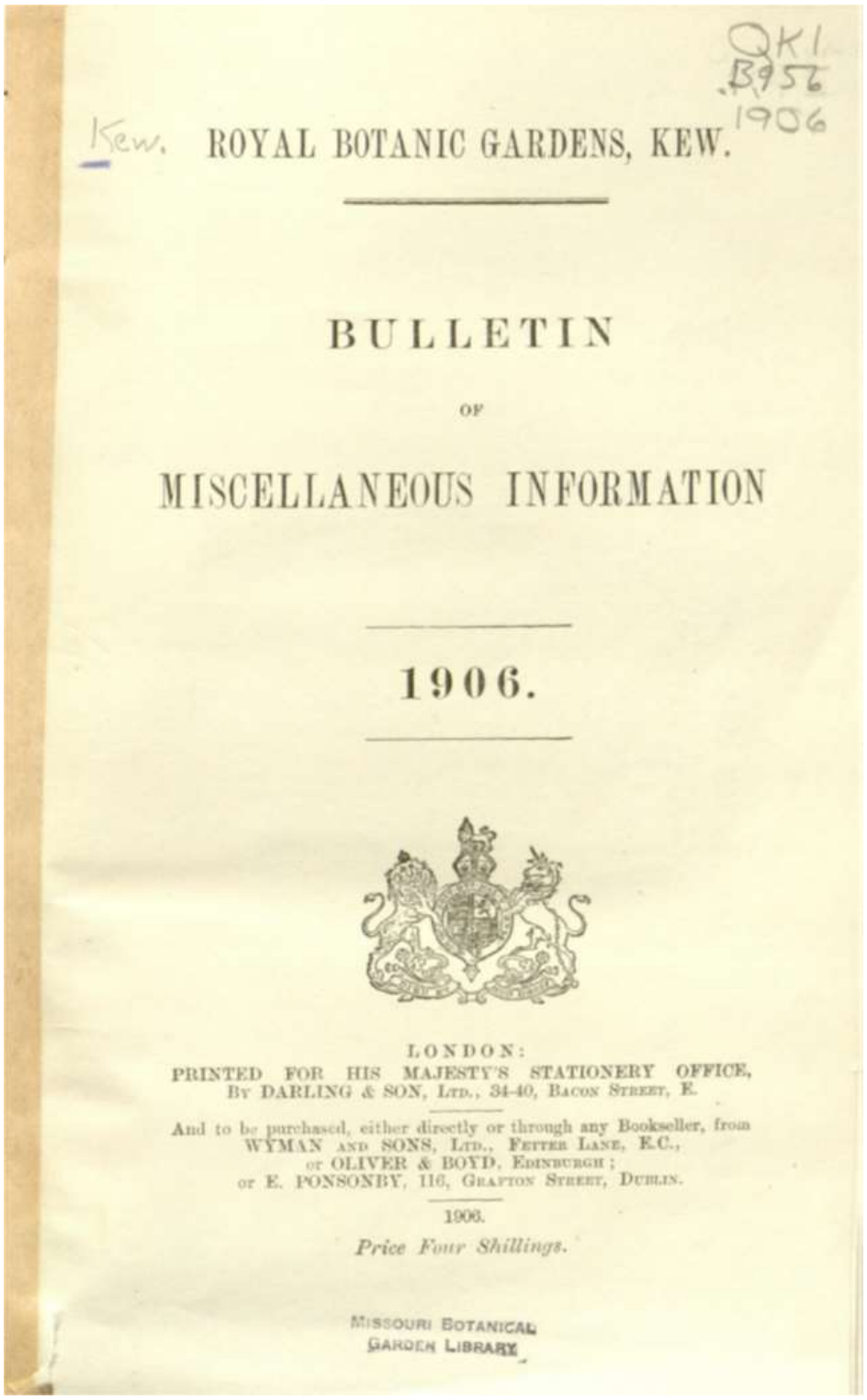
ATTACH



Kew. ROYAL BOTANIC GARDENS, KEW.

BULLETIN

OF

MISCELLANEOUS INFORMATION

1906.

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1906.

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Attach 1. Excerpt of the original description of the accepted name "*Cymbopogon citratus*" in the *Bulletin of Miscellaneous Information* (Royal Botanical Gardens, Kew, 1906).

Practically nothing is known of the conditions under which this grass grows; but it has a distinctly xerophytic habit. It is a highly aromatic grass. There is, however, no evidence that it is used for extracting oil or for any other purposes, unless it is one of the 'lemon-grasses' of the Malabar district to which the following* refers: "The natives of Ernad and Waluvanad empirically distinguish no fewer than 27 species of lemon-grass, but say that only five of these varieties possess a commercial value. They also state that the most valuable of these varieties does not blossom. Ernad and Waluvanad, I am reliably informed, are full of hills on which lemon-grass grows wild and could be had virtually for the collecting." The variety which does not blossom is, I may add, very probably *C. citratus*. The vernacular names which I have been able to collect require further confirmation and revision. They are to be found in the second part of this paper under *C. coloratus*.

7. Cymbopogon citratus, *Stapf.*

(*Andropogon citratus DC.*)

Lemon Grass (*κατ' ἐξοχήν*); Sereh (Malay).

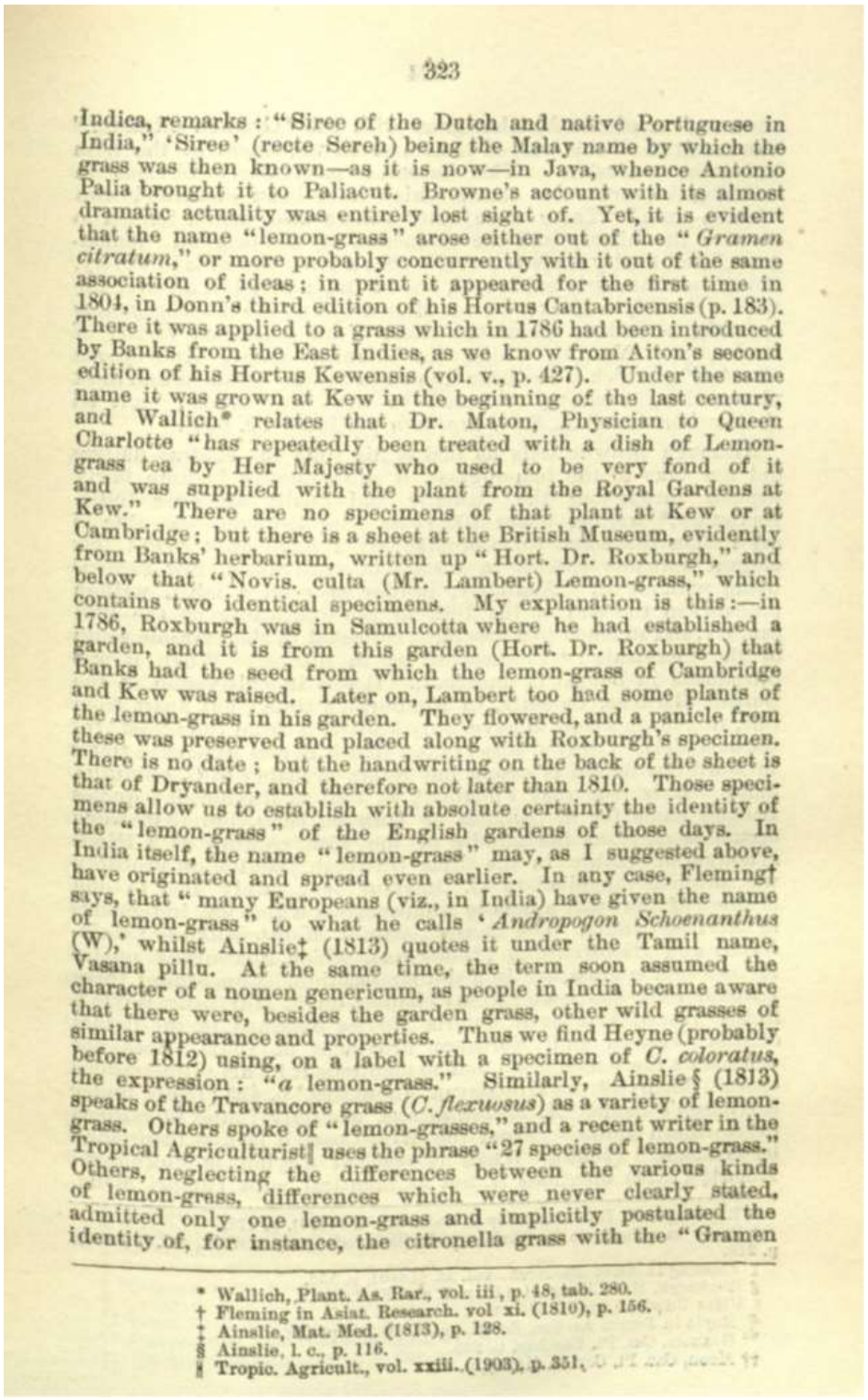
EARLY HISTORY IN INDIA.—In 1695, Petiver announced in his 'Museum' (p. 55, no. 586) a "Gramen *citratum* fragrantissimum e Madraspatan." A few years later Plukenet referred to the same grass in Almag. Mant. p. 97 (1700) in these words: "Gramen cyperoides citratum, Ind. Or. foliis odore corticum Citri; Vasnapillee Malabarorum." The sender of the grass was Dr. Samuel Browne, Surgeon at Fort St. George, and he, in his 'Seventh Book of East Indian Plants,'† edited and commented on by Petiver, gives the following account of it: "This is a most delicate sort of *fragrant Grass* which being rubbed smells like *Baume* and *Lime* or *Limon peel* together. The *Portuguese Women* fume their children with it, and give the *Decoction* of it with other things for *Fevers* and to strengthen weak stomachs: but the Natives use it not, which together with its growing in *Gardens* on the Sea coast and not up the Country, as I can yet observe, makes me think the Portuguese brought this from other parts and planted it here; certainly, so excellent a Plant of such *Fragrant* and *Aromatik taste* must have many *Vertues*. I use it in many cases, and generally with success. While I was writing this, in came a Person, who says, that about 30 years ago, viz., about 1666, one Antonio Palia, brought 3 Pots of this Grass from Batavia to Paliacut, one of which he sent to a Garden, here at Madrass." Browne's specimen is no longer in existence; but there can be no doubt as to what the plant was. From a note on a label in Rottler's herbarium, we know that the Portuguese in India called it 'Herba cheirosa,' the Portuguese equivalent of the Tamil Vasana-pillu, and Roxburgh in an early manuscript‡ of his Flora

* Reprint from the Madras Mail in Tropic. Agricult., vol. xxiii. (1903), p. 351.

† Petiver, Samuel Browne, his seventh Book of East Indian Plants, in Phil. Trans., vol. xxiii. (1702), pp. 1251–1252.

‡ This manuscript was long in the possession of the Govan family and is now at Kew. It is, as compared with the published "Flora Indica," much abridged.

Attach 1. (cont.).



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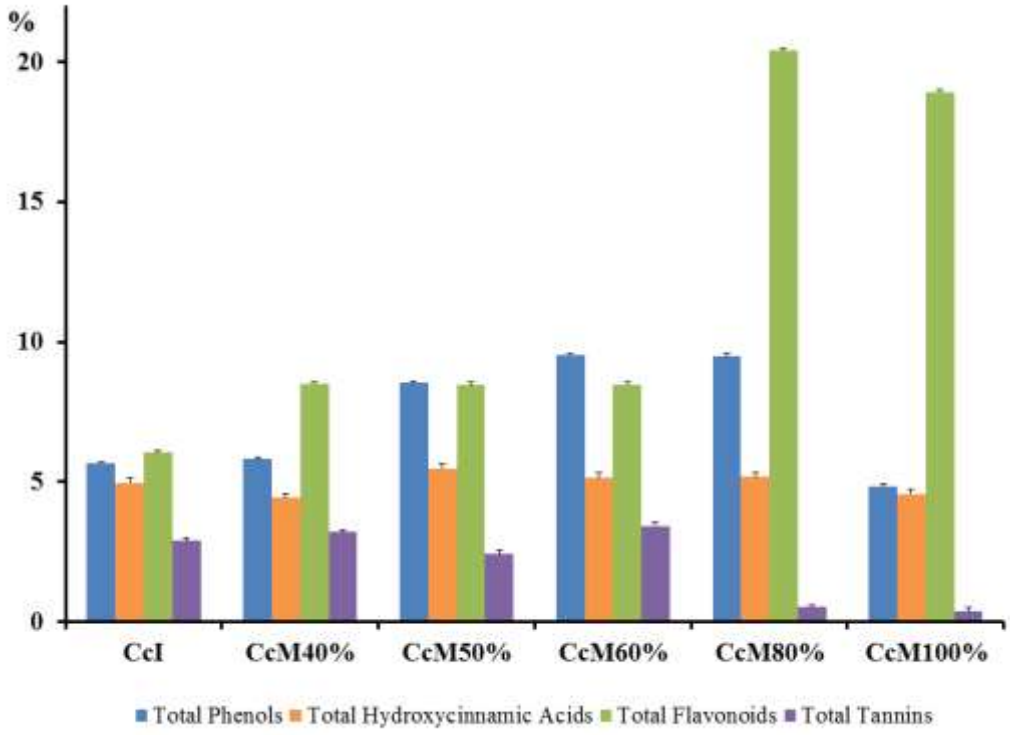
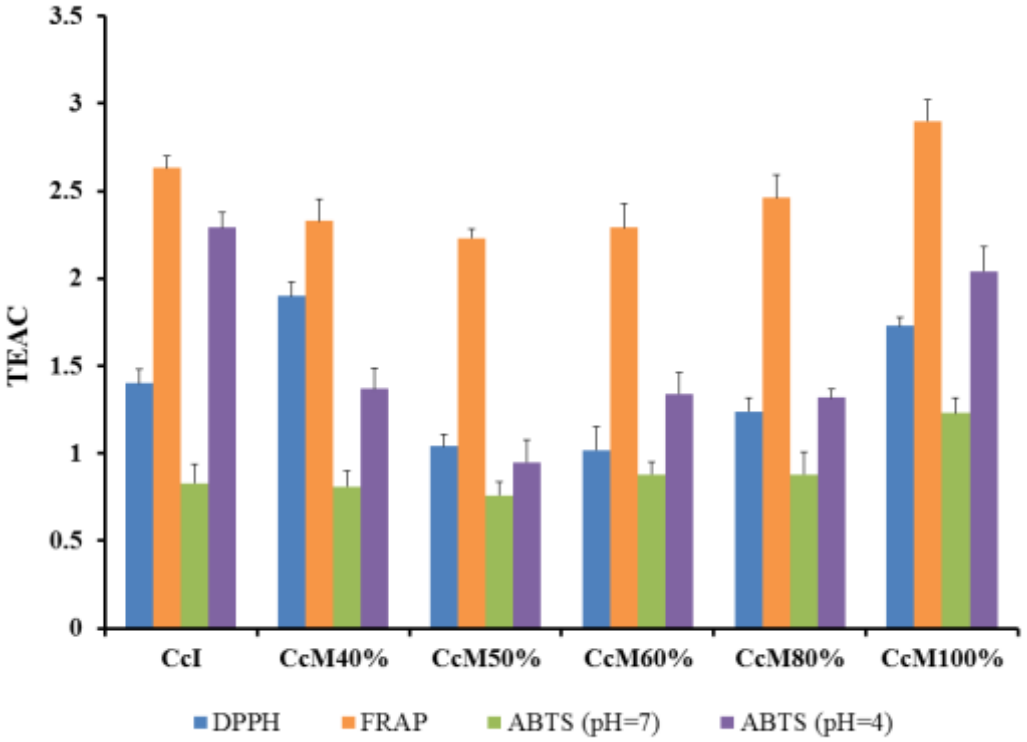
Indica, remarks : "Siree of the Dutch and native Portuguese in India," 'Siree' (recte Sereh) being the Malay name by which the grass was then known—as it is now—in Java, whence Antonio Palia brought it to Paliacut. Browne's account with its almost dramatic actuality was entirely lost sight of. Yet, it is evident that the name "lemon-grass" arose either out of the "*Gramen citratum*," or more probably concurrently with it out of the same association of ideas; in print it appeared for the first time in 1804, in Donn's third edition of his Hortus Cantabricensis (p. 183). There it was applied to a grass which in 1786 had been introduced by Banks from the East Indies, as we know from Aiton's second edition of his Hortus Kewensis (vol. v., p. 427). Under the same name it was grown at Kew in the beginning of the last century, and Wallich* relates that Dr. Maton, Physician to Queen Charlotte "has repeatedly been treated with a dish of Lemon-grass tea by Her Majesty who used to be very fond of it and was supplied with the plant from the Royal Gardens at Kew." There are no specimens of that plant at Kew or at Cambridge; but there is a sheet at the British Museum, evidently from Banks' herbarium, written up "Hort. Dr. Roxburgh," and below that "Novis. culta (Mr. Lambert) Lemon-grass," which contains two identical specimens. My explanation is this:—in 1786, Roxburgh was in Samulcotta where he had established a garden, and it is from this garden (Hort. Dr. Roxburgh) that Banks had the seed from which the lemon-grass of Cambridge and Kew was raised. Later on, Lambert too had some plants of the lemon-grass in his garden. They flowered, and a panicle from these was preserved and placed along with Roxburgh's specimen. There is no date; but the handwriting on the back of the sheet is that of Dryander, and therefore not later than 1810. Those specimens allow us to establish with absolute certainty the identity of the "lemon-grass" of the English gardens of those days. In India itself, the name "lemon-grass" may, as I suggested above, have originated and spread even earlier. In any case, Fleming† says, that "many Europeans (viz., in India) have given the name of lemon-grass" to what he calls '*Andropogon Schoenanthus* (W),' whilst Ainslie‡ (1813) quotes it under the Tamil name, Vasana pillu. At the same time, the term soon assumed the character of a nomen genericum, as people in India became aware that there were, besides the garden grass, other wild grasses of similar appearance and properties. Thus we find Heyne (probably before 1812) using, on a label with a specimen of *C. coloratus*, the expression: "*a* lemon-grass." Similarly, Ainslie§ (1813) speaks of the Travancore grass (*C. flexuosus*) as a variety of lemon-grass. Others spoke of "lemon-grasses," and a recent writer in the Tropical Agriculturist‖ uses the phrase "27 species of lemon-grass." Others, neglecting the differences between the various kinds of lemon-grass, differences which were never clearly stated, admitted only one lemon-grass and implicitly postulated the identity of, for instance, the citronella grass with the "Gramen

* Wallich, Plant. As. Rar., vol. iii, p. 48, tab. 280.
† Fleming in Asiat. Research. vol xi. (1810), p. 156.
‡ Ainslie, Mat. Med. (1813), p. 128.
§ Ainslie, l. c., p. 116.
‖ Tropic. Agricult., vol. xxiii. (1903), p. 351.

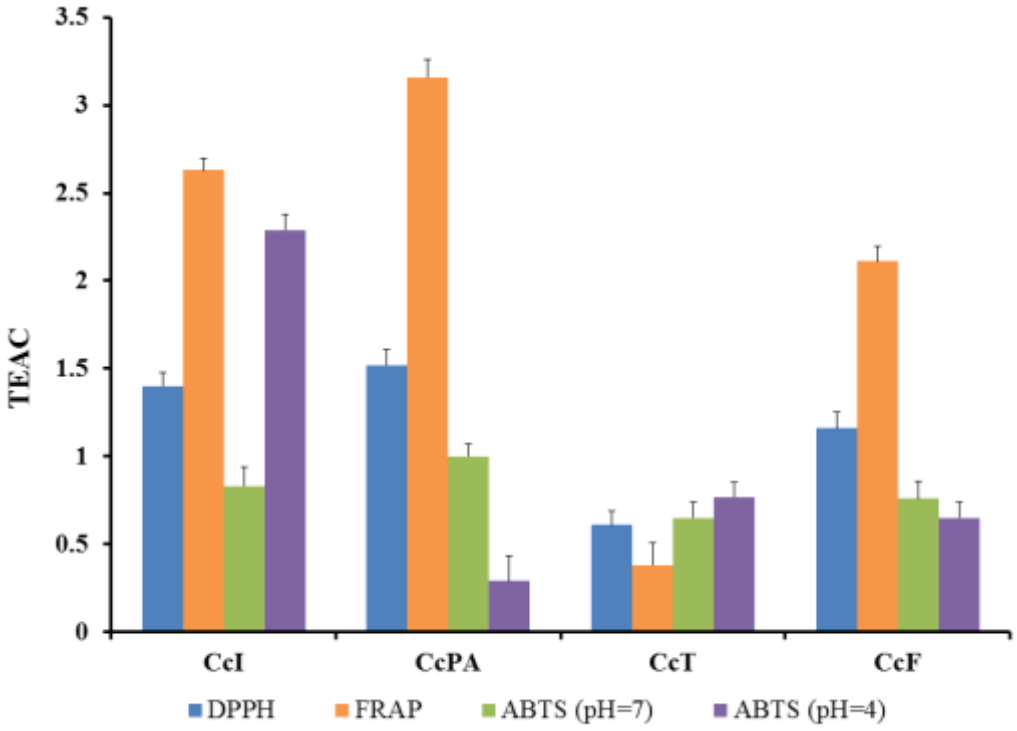
Attach 1. (cont.).



Attach 2. Phenolic compounds amount (%, g of respective standard equivalents per 100 g of extract) quantified in lemongrass infusion (**CcI**), 40% ethanolic (**CcM40%**), 50% ethanolic (**CcM50%**), 60% ethanolic (**CcM60%**), 80% ethanolic (**CcM80%**) and absolute ethanolic (**CcM100%**) macerates. Results are expressed as mean ± SEM (n=3).



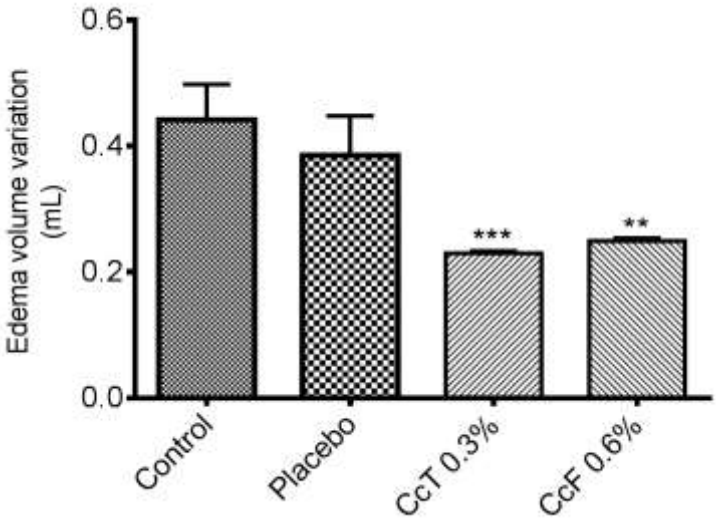
Attach 3. Antioxidant activity (expressed in TEAC value) of lemongrass infusion (**CcI**), 40% ethanolic (**CcM40%**), 50% ethanolic (**CcM50%**), 60% ethanolic (**CcM60%**), 80% ethanolic (**CcM80%**) and absolute ethanolic (**CcM100%**) macerates. Results are expressed as mean ± SEM (*n*=3).



Attach 4. Antioxidant activity (expressed in TEAC value) of lemongrass infusion (**CcI**), CcI phenolic acids fraction (**CcPA**), CcI tannins fraction (**CcT**) and CcI flavonoids fraction (**CcF**). Results are expressed as mean ± SEM (*n*=3).

Attach 5. *In vivo* anti-inflammatory activity of diferente topical formulations of CcI, by carrageenan-induced oedema reduction in rats.

Formulation type	**HydroGel**	**O/W Emulsion**	**O/W Emulsion**	**O/W Emulsion**
Formula	Carbopol 940	Stearic acid	Stearic acid	Stearic acid
	Triethanolamine	Triethanolamine	Triethanolamine	Triethanolamine
	Glycerol	Glycerol	Glycerol	Glycerol
	Water	Water	Water	Water
	Isopropanol	-	Propylene glycol	Propylene glycol
	-	-	-	Oleic acid
	CcI 4%	CcI 4%	CcI 4%	CcI 4%
Oedema reduction (%)	12	31	41	47



Attach 6. Effect of topical application of the placebo, positive control (diclofenac sodium, 1%), CcT 0.3% and CcF 0.6% formulations on the oedema volume induced by 1% carrageenan, 4 h after inflammation induction. Each result represents the mean ± SEM (n = 6/group). **p<0.01 and ***p<0.001, compared with the control group.