Maria Eduarda da Rocha Pereira Dias Moita

Effects of *Echium plantagineum* L. bee pollen on macrophages and basophils: metabolic profile *vs* inflammatory mediators, degranulation and oxidative stress

Thesis for Doctor Degree in Pharmaceutical Sciences

Phytochemistry and Pharmacognosy Speciality

Work performed under the supervision of

Professor Doctor Paula Cristina Branquinho de Andrade

And co-supervision of

Professor Doctor Patrícia Carla Ribeiro Valentão

Doctor Luís Manuel Lopes Rodrigues da Silva

Porto 2015

To Miguel, Daniel and Tiago

Knowledge comes, but wisdom lingers

A. Tennyson

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2. **Moita E**, Sousa C, Andrade PB, Fernandes F, Pinho BR, Silva LR, Valentão P. Effects of *Echium plantagineum* bee pollen on basophil degranulation: relationship with metabolic profile. Molecules 2014; 19: 10635-49.

3. Sousa C, **Moita E**, Valentão P, Fernandes F, Monteiro P, Andrade PB. Effects of colored and non-colored phenolics of *Echium plantagineum* L. bee pollen in Caco-2 cells under oxidative stress induced by *tert*-butyl hydroperoxide. J. Agric. Food Chem. 2015; 63: 2083-91

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Poster communications

1. **Moita E**, Sousa C, Monteiro P, Valentão P, Andrade PB. Compostos fenólicos e ação antioxidante de pólen de abelha de *Echium plantagineum* L.. **XX Encontro Luso-Galego de Química**, 26-28 November 2014, Porto (Portugal).

2. Monteiro P, **Moita E**, Sousa C, Valentão P, Andrade PB. Effects of coloured and non-coloured phenolics of *Echium plantagineum* L. bee pollen in Caco-2 cells. **IJUP'14 – 7th Meeting of Young Researchers of U.Porto**, 12-13 February 2014, Porto (Portugal).

3. **Moita E**, Silva LR, Valentão P, Sousa C, Andrade PB. Biological activities of *Echium plantagienum* L. pollen hydro-alcoholic extract. **IJUP'12 – 5th Meeting of Young Researchers of U.Porto,** 22-24 February 2012, Porto (Portugal).

AUTHOR'S DECLARATION

The author states to have afforded a major contribution to the conceptual design, technical execution of the work, interpretation of the results and manuscript preparation of the published work included in this dissertation.

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The European Union (FEDER funds through COMPETE) and National Funds (FCT, Fundação para a Ciência e Tecnologia) through project Pest-C/EQB/LA0006/2013 and from the European Union (FEDER funds) under the framework of QREN through Project NORTE-07-0124-FEDER-000069.

ABSTRACT

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Bee-collected pollen has been used as a folk medicine and foodstuff for human consumption for centuries. Even today it is very sought after for its therapeutic and nutritional properties.

On the other hand, several classes of bee pollen components have been used to establish quality parameters of pollen, to characterize it in terms of botanical origin, to recognize taxonomic markers and to evaluate their nutritional and biological properties.

Oxidative stress and inflammation play important roles in disease development. This dissertation intended to evaluate the anti-inflammatory and anti-allergic potential of *Echium plantagineum* L. bee pollen to support its claimed health beneficial effects.

The hydromethanol extract efficiently scavenged nitric oxide radical (•NO), although against superoxide anion ($O_2^{\bullet -}$) it behaved as antioxidant at lower concentrations and as pro-oxidant at higher concentrations. The anti-inflammatory potential was evaluated in LPS-stimulated macrophages. The levels of •NO and L-citrulline decreased for all extract concentrations tested, while the levels of prostaglandins, their metabolites and isoprostanes, evaluated by UPLC-MS, decreased with low extract concentrations. So, *E. plantagineum* bee pollen can exert anti-inflammatory activity by reducing •NO and prostaglandins.

The anti-allergic potential of the hydromethanol extract obtained from *E. plantagineum* bee pollen was also evaluated, by assessing β-hexosaminidase release in rat basophilic leukemic cells (RBL-2H3). Two different stimuli were used: calcium ionophore A23187 and IgE/antigen complex. Lipoxygenase inhibitory activity was evaluated in a cell-free system.

In RBL-2H3 cells stimulated with calcium ionophore or IgE/antigen, the hydromethanol extract significantly decreased β-hexosaminidase release until the concentration of 2.08 mg mL^{-1}, without compromising cellular viability. No effect was found on lipoxygenase. Overall, this study showed promising results, substantiating for the first time the utility of the intake of *E. plantagineum* bee pollen to prevent allergy and ameliorate allergy symptoms, although a potentiation of an allergic response can occur, depending on the dose used.

The hydromethanol extract was analysed for its phenolic content, as it is known that these are important bioactive compounds. The chromatogram obtained by HPLC-DAD showed kaempferol-3-*O*-neohesperidoside as a major peak, followed by its acylated derivative, kaempferol-3-*O*-(3'/4'-acetyl)-neohesperidoside, and eight minor peaks

corresponding to quercetin and kaempferol derivatives. In addition, HPLC-UV and GC-IT/MS methods were used to determine organic acids and fatty acids, respectively. Eight

organic acids and five fatty acids were determined for the first time, malonic acid and α-linolenic acid being the main compounds from each class. This extract revealed to be devoid of alkaloids.

To widen the knowledge in *E. plantagineum* bee pollen, two fractions of pollen's acidified methanol extract, enriched either in flavonols or anthocyanins, were characterized by HPLC-DAD and their antioxidant properties were evaluated in Caco-2 cells subjected to oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BHP). For comparison purposes the whole extract was also studied.

Seven flavonols and five anthocyanins were detected in both whole extract and fraction II, while fraction I contained six flavonols (in higher amounts than in fraction II) and small amounts of petunidin-3-*O*-rutinoside. The pre-exposition to fraction I imparted a tendency to protect cells, while fraction II and the whole extract aggravated the toxicity of *t*-BHP at some of the tested concentrations.

The protective effects appeared to be correlated with the levels of total glutathione, while there was no observable correlation between cellular viability and reactive species. No significant effect on antioxidant enzymes activity was noticed. Overall, anthocyanins seemed to abrogate the antioxidant potential of flavonoid-rich *E. plantagineum* bee pollen extract.

E. plantagineum bee pollen proved to be nutritionally and pharmacologically interesting, due to its chemical composition and to the biological activity of its constituents, namely phenolic compounds. This study contributes to increase the possibility of commercialization of this natural product in the granular form or as an extract.

Keywords: *Echium plantagineum* L. bee pollen; metabolic profile; inflammation; allergy; oxidative stress.

RESUMO

RESUMO

O pólen de abelha tem sido usado há séculos como remédio tradicional e alimento para consumo humano. Ainda hoje é muito procurado pelas suas propriedades nutritivas e terapêuticas.

Por outro lado, várias classes de compostos do pólen de abelha têm sido utilizadas para se estabelecer os seus parâmetros de qualidade, para a sua caracterização em termos de origem botânica, reconhecimento de marcadores taxonómicos e avaliar as suas propriedades biológicas e nutricionais.

A inflamação e o stress oxidativo desempenham um papel importante no desenvolvimento de várias doenças. Nesta dissertação pretendeu-se avaliar o potencial anti-inflamatório e antialérgico do pólen de abelha de *Echium plantagineum* L., para suporte científico dos seus aclamados efeitos benéficos para a saúde.

O extrato hidrometanólico demonstrou capacidade para sequestrar o óxido nítrico ($^{\bullet}NO$); no entanto, relativamente ao radical anião superóxido ($O_2^{\bullet-}$) comporta-se como antioxidante em baixas concentrações e como pró-oxidante em concentrações mais altas. O potencial anti-inflamatório foi avaliado em macrófagos estimulados com LPS. Os níveis de $^{\bullet}NO$ e de L-citrulina diminuíram com todas as concentrações de extrato testadas, enquanto os níveis de prostaglandinas, os seus metabolitos e isoprostanos, analisados por UPLC-MS, diminuíram com baixas concentrações de extrato. Assim, o extrato de pólen de abelha de *E. plantagineum* exerce atividade anti-inflamatória reduzindo os níveis de $^{\bullet}NO$ e de prostaglandinas.

O potencial anti-alérgico do extrato hidrometanólico de pólen de abelha de *E. plantagineum* foi, também testado relativamente ao seu efeito sobre a libertação de β-hexosaminidase, em basófilos leucémicos de ratinho (RBL-2H3). Foram usados dois estímulos diferentes: o ionóforo de cálcio A23187 e o complexo IgE/antigénio. A atividade inibitória da lipoxigenase foi analisada em sistema não celular.

O extrato hidrometanólico diminuiu significativamente a libertação de β-hexosaminidase das células RBL-2H3 estimuladas com ionóforo de cálcio ou IgE/antigénio até à concentração de 2,08 mg mL^{-1}, sem comprometer a viabilidade celular. Não foi observado qualquer efeito na lipoxigenase. Globalmente, foram obtidos resultados promissores que substanciam, pela primeira vez, a utilidade do consumo de pólen de abelha de *E. plantagineum* para prevenir a alergia e melhorar sintomas de alergia, apesar de poder ocorrer uma potenciação da resposta alérgica, dependendo da dose usada.

O extrato hidrometanólico foi analisado relativamente à sua composição fenólica. O cromatograma obtido por HPLC-DAD mostrou o canferol-3-*O*-neoesperidósido como composto principal, seguido de um seu derivado acilado, canferol-3-*O*-(3'/4'-acetil)-neo-hesperidósido, além de oito compostos em menor quantidade, correspondentes a derivados de quercetina e de canferol. Adicionalmente, o extrato foi analisado por HPLC-UV e por GC-IT/MS para pesquisa de ácidos orgânicos e de ácidos gordos, respetivamente. Oito ácidos orgânicos e cinco ácidos gordos foram determinados pela primeira vez, sendo o ácido malónico e o ácido α-linolénico os compostos principais de cada classe. Este extrato não apresentou alcaloides na sua composição.

Para alargar o conhecimento do pólen de abelha de *E. plantagineum* foram caracterizadas por HPLC-DAD duas frações do extrato metanólico adicificado, ricas em flavonóis ou em antocianinas, cuja capacidade antioxidante foi avaliada em células Caco-2 sob stress oxidativo induzido pelo *terc*-butil-hidroperóxido (*t*-BHP). O extrato inteiro foi usado para comparação.

Na fração II e no extrato total foram identificados sete flavonóis e cinco antocianinas, enquanto a fração I continha seis flavonóis (em concentração superior à da fração II) e pequenas quantidades de petunidina-3-*O*-rutinósido. Da pré-exposição à fração I foi possível observar uma tendência para proteger as células, enquanto a fração II e o extrato total agravaram a toxicidade do *t*-BHP em algumas das concentrações testadas.

Os efeitos protetores pareceram estar correlacionados com os níveis de glutationa total, não se tendo observado qualquer correlação entre a viabilidade celular e as espécies reativas. Os extratos não demonstraram ter efeito significativo na atividade das enzimas antioxidantes. Em geral, as antocianinas pareceram anular o potencial antioxidante do extrato metanólico acidificado do *E. plantagineum* rico em flavonóis.

O pólen de abelha do *E. plantagineum* revelou ser interessante sob o ponto de vista nutricional e farmacológico, devido à sua composição química e à atividade biológica dos seus constituintes, principalmente dos seus compostos fenólicos. Este estudo contribui para aumentar a possibilidade de comercialização deste produto natural, na forma de granulado ou como um extrato.

Palavras–chave: Pólen de abelha de *Echium plantagienum* L; perfil metabólico; inflamação; alergia; stress oxidativo.

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ABBREVIATIONS AND SYMBOLS

AA	Arachidonic acid
ALA	α-Linolenic acid
BSA	Albumin from bovine serum
CoA	Coenzyme A
COX	Cyclooxygenase
CE	Capillary electrophoresis
DAD	Diode array
DCFH-DA	2′,7′-Dichlorofluorescein diacetate
dehydroPA	1,2-Dehydropyrrolizidine ester alkaloid
DGLA	Dihomo-γ-linolenic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	DL-Dithiothreitol
EO	*Echium* oil
EBSS	Earle's Balanced Salt Solution
EPA	Eicosapentaenoic acid
ESI	Electrospray ionisation
FAME	Fatty acid methyl esters
FBS	Foetal bovine serum
FGT	Flavonoid 3-*O*-glucosyltransferase
FLS	Flavonol synthase
GC	Gas chromatography
GC-MS	Gas chromatography coupled with mass spectrometry
GLA	γ-Linolenic acid

HBS	Hepes Buffered Saline
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
HPLC-DAD-ESI/MS^n	High-performance liquid chromatography-diode array detection-electrospray ionisation/ multi-stage mass spectrometry
HS	Homospermidine synthetase
IgE	Immunoglobulin E
iNOS	Inducible nitric oxide synthase
IPNI	The International Plant Names Index
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxin
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
MS	Mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
MTT	(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide reduced form
NBT	Nitroblue tetrazolium
$^{\bullet}NO$	Nitric oxide radical
OA	Oleic acid
$O_2^{\bullet-}$	Superoxide anion radical

PA	Pyrrolizidine alkaloid
PAL	Phenylalanine ammonia-lyase
PALMs	Pollen-associated lipid mediators
PANO	Pyrrolizidine *N*-oxides
PBS	Phosphate buffer saline
PG	Prostaglandin
PLA2	Phospholipase A2
PMS	Phenazine methosulfate
PUFA	Polyunsaturated fatty acid
RP-C18	Reversed-phase C18 column
RP-HPLC	Reversed-phase high-performance liquid chromatography
*R*t	Retention time
SDA	Stearidonic acid
SNP	Sodium nitroprusside
SPE	Solid-phase extraction
tert-BHP	*tert*-Butylhydroperoxide
THF	Tetra-hydrofuran
TNB	5-thio-2-nitrobenzoic acid
TX	Thromboxane
UPLC	Ultra performance liquid chromatography
UPLC-QqQ-MS/MS	Ultra performance liquid chromatography coupled with mass spectrometry
UV	Ultraviolet

DISSERTATION OUTLINE

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The present dissertation is divided into five main sections:

Chapter I – Introduction

In chapter I a general overview on the thesis subject and objectives is included. A review on the existing literature about the different topics approached in this dissertation is provided. It affords a basis to understand the objectives and the obtained results.

Chapter II – Experimental section

This section provides detailed information about all the methods and techniques used for the realization of all the works presented in this dissertation.

Chapter III – Results and discussion

This section is divided in two main parts. The first part is related with the studies conducted with *Echium plantagineum* L. bee pollen hydromethanolic extract, namely the evaluation of its anti-inflammatory and anti-allergic potentials. The second one concerns to the study of the acidified methanol extract of the same matrix, relating its composition and the protective effect against oxidative stress. All of the results are integrated and discussed concerning their relevance, linking them to the existing scientific reports.

Chapter IV – Conclusions and perspectives

The main conclusions that can be taken from the developed work are summarized in this section. In addition, some future perspectives are approached.

Chapter V – References

In this last section are listed all the bibliographic references used in this dissertation.

Chapter I

Introduction

1. *Echium plantagineum* Linnaeus

1.1. General considerations

According to the "Taxonomic Nomenclature Checker Germplasm Resources Information Network" (TNC-GRIN), the *Echium* genus comprises twenty-four species. Other taxonomic databases, namely from the "International Association for Plant Taxonomy" (IAPT), the "International Plant Names Index" (IPNI) and the "International Global Plant Checklist" (IOPI) describe a long list of *Echium* species.

The *Echium* genus (Boraginaceae) contains two distinctive nodes: the predominantly woody species of the macaronesian archipelago, and the herbaceous species that are chiefly distributed in Europe and North Africa (1). Within the latter group, the main centres of species diversity are the Iberian Peninsula, with about sixteen species, and North-West Africa (particularly Morocco) presenting about twenty species (1).

In this dissertation *Echium plantagineum* L. was considered, which characteristics are provided below.

1.2. Characteristics

1.2.1. Habitat

E. plantagineum is a widespread weed in warm-temperate regions. It is found mostly in areas dominated by winter rainfalls and can flourish in a wide range of soils (2). Native from western Mediterranean Basin, it extends into central Europe and locally naturalized in northern Europe (1).

Since its introduction in Australia, *E. plantagineum* has spread to infest vast areas of predominantly agricultural land in south-east and south-west Australia, where it can be the dominant pasture species (3).

1.2.2. Stems and leaves

E. plantagineum is an annual herb, occasionally biennial, that grows to 1.5 m high, but commonly 30 to 60 cm. It is an erect softly hairy species, with several flowering stems, that reproduces by seed (1, 3). Young plants form rosettes with basal leaves that can be up to 25 cm long, broadly ovate, petiolate, with prominent lateral veins. As it grows, it produces several stems starting at the base of the plant, although it can occasionally be

found to be single stemmed. The stems have stout white hairs. The stem leaves are narrower and smaller, sessile and cordate at the base (2, 4).

1.2.3. Flowers and fruit

The inflorescence (**Figure 1**) is usually branched. The calyx is 7-10 mm at anthesis, up to 15 mm in the fruit. The corolla is 18-30 mm, trumpet shaped, curled and purple, and unrolls as the flowers open; it is hairy only on the veins and margins, usually with two or less exserted stamens (2, 4). The flowers are sessile, each one consisting of five petals that are 2-3 cm long and five stamens, two being longer than the others and projecting significantly from the corolla. Its filaments are rather sparsely hairy and the pollen is bluish (2). The fruit consists of four nutlets 2.5 x 2 mm, tuberculate, pale brownish-grey, surrounded by persistent stiffly bristled calyx (2, 4).

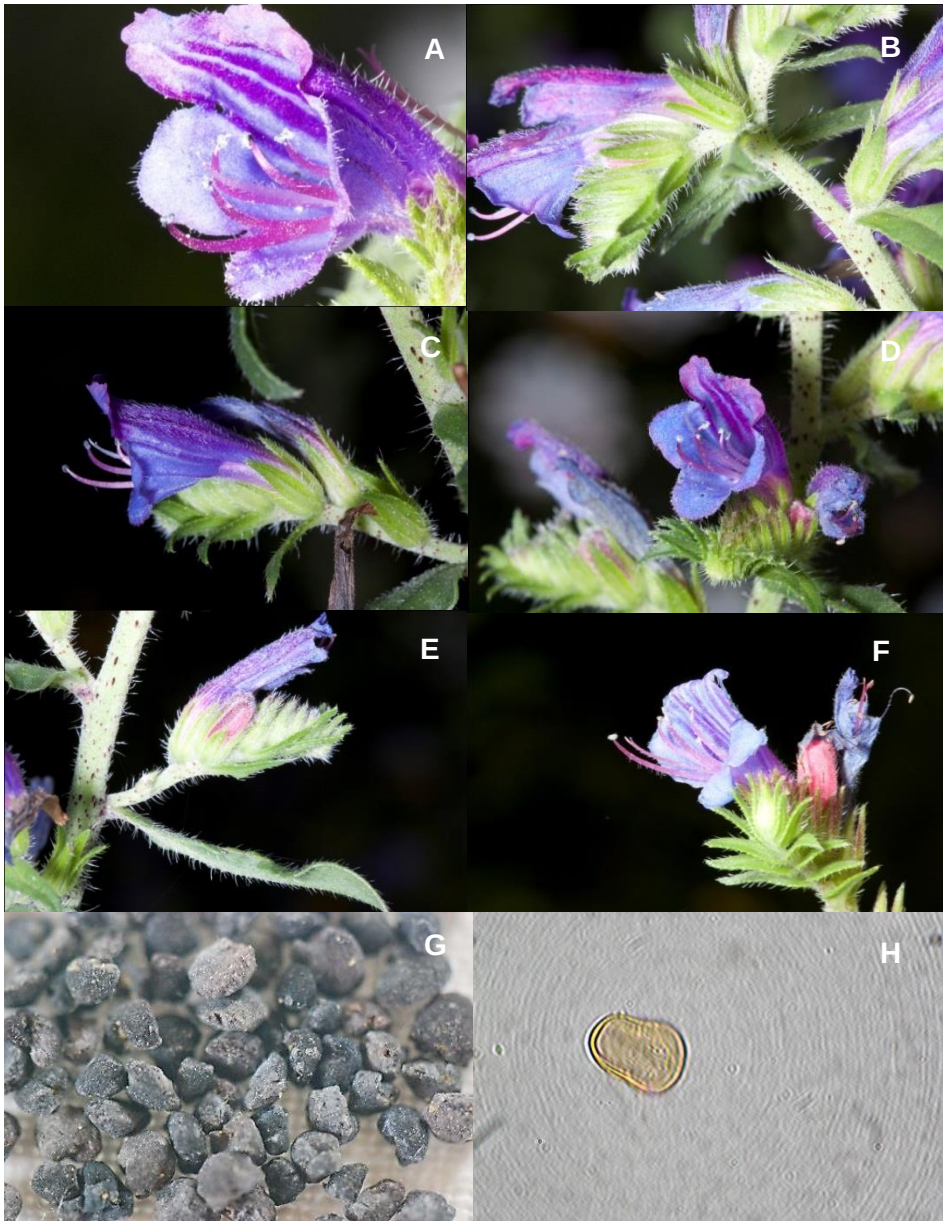


Figure 1. Several aspects of *Echium plantagineum* plant: A – Corolla with five stamens and one stigma, five petals; B – Stem with stout white hairs; C – Purple colour of the corolla, two stamens protruding, hence the common name “Purple Viper’s Bugloss”; D – Alternate flowers; E – Hairy leaf without petiole; F – Flowers in three stages of development; G – Pollen with the characteristic blue colour; H – Pollen´s microscopic analysis.
(Photographs by Eduarda Moita, António Moita and Sérgio Moreira, Paredes de Coura, June 2011 and July 2014; different magnifications).

1.3. Distribution of *E. plantagineum* in Portugal

E. plantagineum has a general distribution in the country, except in Alentejo coast (Serra do Cercal) and in Serra Algarvia (Serra de Monchique and Serra Espinhaço de Cão). It concentrates mainly in Alto Alentejo (Évora region) (**Figure 2**). In Portugal it is known as “Chupa-mel”, “Língua-de-vaca”, “Soagem” or “Soagem viperina”.

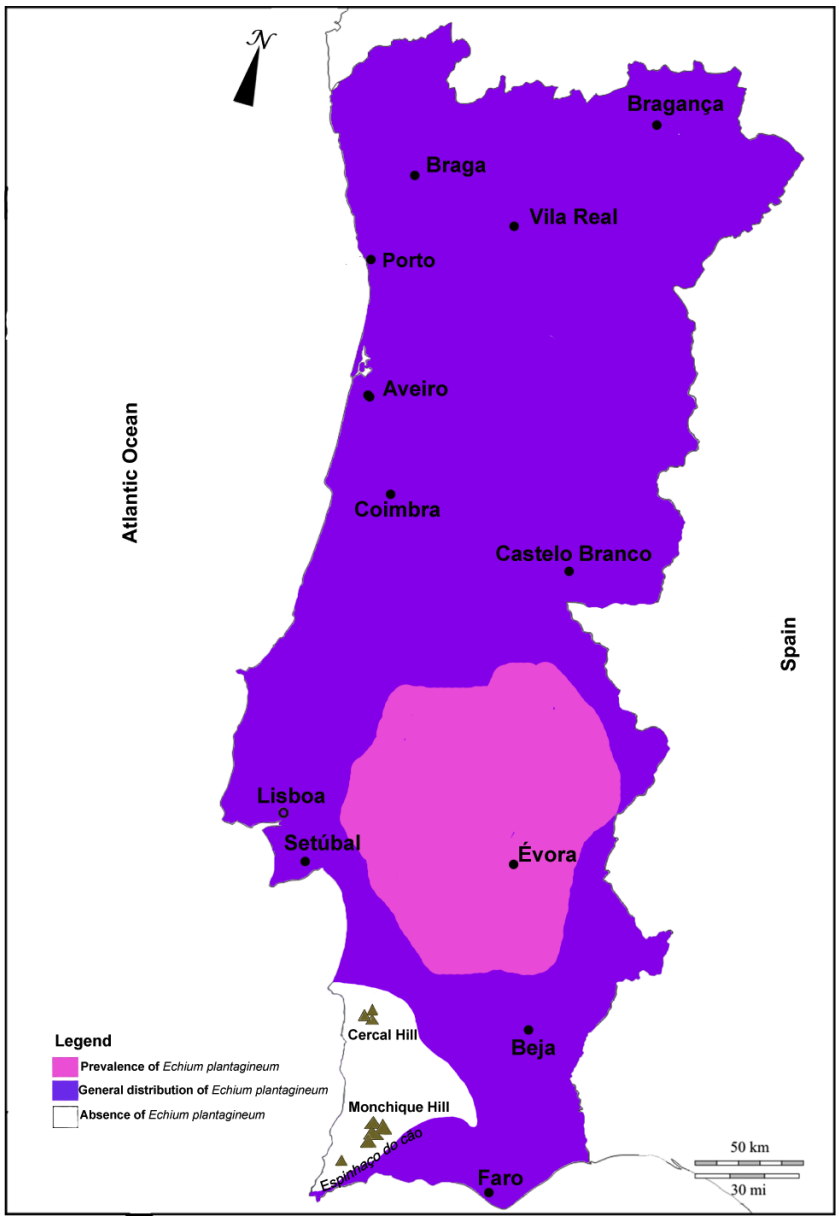


Figure 2. Geographical distribution of *E. plantagineum* in Portugal [data from (5)].

The habitat includes cultivated and ruderal areas, the budding season occurring in March-June. It is a therophyte and hemicryptophyte plant (4).

2. Secondary metabolism

Plants have the ability to synthesize an enormous variety of secondary metabolites, since they need to respond to a continuously changing and often hostile environment, in order to survive and reproduce (6).

Environmental factors, such as nutrient supply, temperature, light conditions or atmospheric CO_2 concentrations, can influence the levels of carbon-based secondary metabolites in plant tissues and plant partitioning of carbohydrates and energy. As it is generally known that secondary metabolism is linked to primary metabolism by the rates at which the substrates are diverted from primary pathways and funnelled into secondary biosynthetic routes, several environmental factors affecting growth, photosynthesis and other parts of primary metabolism will also affect secondary metabolism (7).

2.1. Alkaloids

Alkaloids are one of the most diverse classes of secondary metabolites found in living organisms, including a wide range of structures resulting from several biosynthetic pathways and with an array of pharmacological activities. Evidence suggests that they may serve specific biological functions. In some plants, the concentration of alkaloids increases just prior to seed formation and then drops off when the seed is ripe, suggesting that alkaloids may play a role in this process. Alkaloids may also protect some plants from destruction by certain insect species (8).

Alkaloids are found in nature in the form of salts (citrates and malates), or combined with tannins (8). In plants they are part of the cell wall (8). They are basic, nitrogenated compounds, with nitrogen atom as part of a heterocyclic system, biosynthesis starting in an amino acid. In **Table 1** are indicated the amino acids that are precursors of alkaloids and the characteristic nuclei they originate (8).

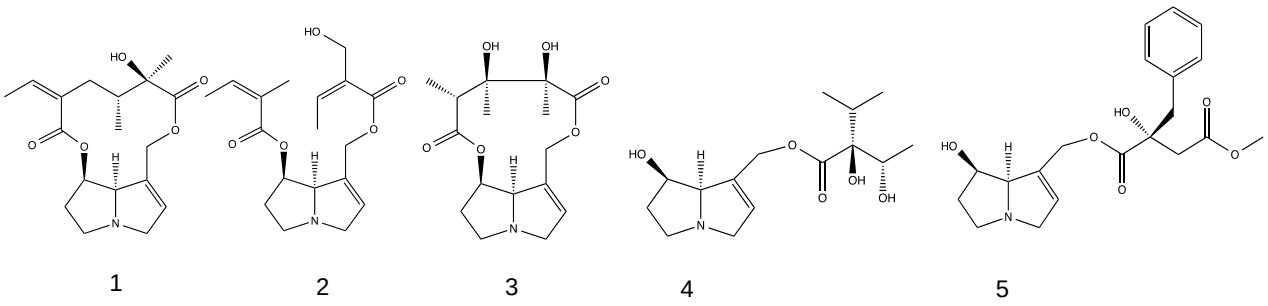
Table 1. Precursor amino acids and origin of the main alkaloid heterocycles.

Precursor amino acid/origin	Heterocycle
Anthranilic acid	Quinoline, quinazoline, benzoxazine
Nicotinic acid	Pyridine
Phenylalanine-Tyrosine	Isoquinoline
Histidine	Imidazole
Lysine	Indolizidine, quinolizidine
Ornithine	Pyrrolidine, pyrrolizidine, tropane
Tryptophan	Indole

The existence of several structural variations is due to allyl oxidations, oxidative coupling, oxidation of aromatic nuclei, esterifications and eterifications. Due to their basic characteristics they give positive reactions in precipitation assays, such as those with Dragendorff's, Mayer's or Bertrand's reagents (8).

2.1.1. Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are constitutively expressed secondary plant defence compounds (9, 10). They comprise about 400 different structures isolated from more than 560 plant species (11), which correspond to ca. 3% of the flowering plants. PAs occur in two major forms, a tertiary form and the corresponding *N*-oxide (12). The occurrence of PAs is restricted to Angiosperms, being limited to the families Boraginaceae (all genera), Asteraceae (Compositae, tribes Senecioneae and Eupatoriae) and Fabaceae (Leguminosae) (genus *Crotolaria*) (12,13). Almost all PAs can be classified into five major structural types (**Figure 3**), all of them being found in Boraginaceae, to which *Echium* genus belongs (10).



1 2 3 4 5

Figure 3. Major structural types of naturally occurring plant pyrrolizidine alkaloids (9): 1 – Senecionine type; 2 – Triangularine type; 3 – Monocrotaline type; 4 – Lycopsamine type; 5 – Phalaenopsine type.

All structures containing a 1,2-double bond are protoxins, being metabolically activated by hepatic Cyt P450 enzymes to toxic pyrroles (14). 1,2-Dehydropyrrolizidine ester alkaloids (dehydroPAs), predominantly found as their *N*-oxides, pyrrolizidine *N*-oxides (PANOs), have been reported in both honey and in pollen obtained directly from the plant and pollen loads collected by bees (11). All of them are considered hazardous if ingested (12).

In PAs the amino alcohols are called necines, the acid moieties being necic acids (15).

2.1.1.1. Biosynthetic pathway

2.1.1.1.1. Biosynthesis of necines

Biosynthesis of PAs starts with the decarboxylation of the amino acids L-arginine and L-ornithine, by the action of arginine and ornithine decarboxylase, respectively, originating putrescine (16). Homospermidine is then formed from two putrescine molecules. This step, the most important one in the biosynthesis of alkaloids, is catalyzed by the specific enzyme homospermidine synthase (HS) (16). Homospermidine is cyclized to the corresponding intermediate iminium ion, which is reduced with further cyclization to the 1-hydroxymethylpyrrolizidines isoretronecanole and trachelanthamidine. Subsequent hydroxylation and dehydration afford retronecine that usually is a basic constituent of toxic pyrrolizidine alkaloids. On the other hand, otonecine, basic component of the otonecine alkaloids, is produced from retronecine, presumably by further hydroxylation and formation of a ketonic group, with simultaneous cleavage of the C-N bond and *N*-methylation (16, 17).

2.1.1.1.2. Biosynthesis of necic acids

Necic acids are mainly composed by L-valine, L-leucine, L-isoleucine and the secondary product of the latter, L-threonine. In contrast with the necine biosynthesis, the synthesis of necic acids follows different routes. Monocarboxylic acids with five carbon atoms, such as angelic, tiglic and sarracinic acid, are generated in the threonine metabolism. Threonine can interact with pyruvate to yield isoleucine, which may be involved in the formation of necic acids. Isoleucine can be degraded to propionyl-Co A and acetyl-Co A *via* tiglyl-CoA (16).

Valine is converted into senecioic, viridifloric and trachelanthic acid by reaction with activated acetaldehyde. The formation of the ten-carbon atoms-containing dicarboxylic acids is only effected by subsequent cyclization of the open-chain necine monocarboxylic acid diesters. Thus, senecionine is produced from diangeloylretronecine, in a reaction similar to the Michael's addition proceeding *via* cationic intermediates (16, 17).

The different metabolic pathways enable a great variability of necic acids. Biosynthesis of dicarboxylic acids takes place in the roots where the alkaloids occur as *N*-oxides. Being available in easily water-soluble form, they are transported to the aerial parts of the plant and stored in vacuoles (18, 19).

2.1.1.2. Biological activities

Pyrrolizidine alkaloids have various interesting biological actions, like antimicrobial activity. Pharmacological and biological effects, e.g., spasmolytic, anti-histaminic, anti-HIV and glucosidase inhibition, have been attributed to some saturated PAs (17). Alkaloid producing species are highly protected from herbivores. They have been referred to as defence agents against both vertebrate and invertebrate predators, as they are reported to be distasteful to them (17).

2.1.1.3. Pyrrolizidine alkaloids in *E. plantagineum*

Several pyrrolizidine alkaloids *N*-oxides have been described in *E. plantagineum*: lepanthine, echimiplatine, 7-*O*-acetyllycopsamine, 7-*O*-acetylintermedine, echimidine, 3'-*O*-acetylechimidine, echiumine and 3'-*O*-acetylechiumine (20).

2.2. Phenolic compounds

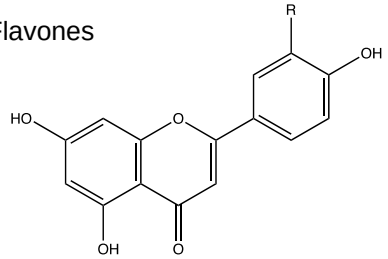
Phenolics represent the most abundant and widely spread class of plant secondary metabolites, comprising a large range of structures (from simple phenolic acids to polymeric molecules with high molecular mass, like condensed or hydrolizable tannins) and functions. They can be classified into water-soluble compounds (phenolic acids, flavonoids and quinones) and water-insoluble compounds (condensed tannins, lignans and cell-wall bound hydroxycinnamic acids) (21, 22). These compounds are synthesized during normal development of the plant and play important functions, including support of the plant body, protection against biotic and abiotic stresses, herbivore deterrence and signalling in plant-plant and plant microbe interactions (21, 22).

Phenolic compounds are thought to be the active ingredients in many dietary plants and traditional medicines used for the treatment of disorders related to oxidative stress and inflammation (22). There is increasing evidence that phenolic compounds present in natural foods may reduce the risk of chronic diseases, such as cancer, inflammation, cardiovascular and neurodegenerative disorders (22). Therefore, there is a growing interest in phenolic compounds of fruits and vegetables, which may promote human health or lower the risk of disease (23).

Phenolic compounds can be classified into distinct families according to their basic molecular structure. Particular emphasis is given here to flavonoids, as the phenolic compounds previously found in *E. plantagineum* belong to this class (**Table 2**) (14, 22).

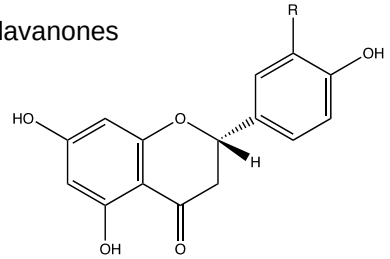
Table 2. Classification of flavonoids according to their basic chemical structure. [adapted from (8)].

Flavones



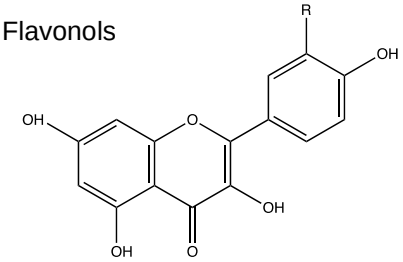
R=H, apigenin
R=OH, luteolin

Flavanones



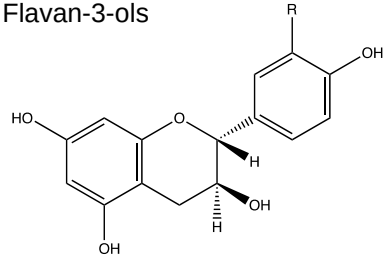
R=H, naringetin
R=OH, eriodictyol

Flavonols



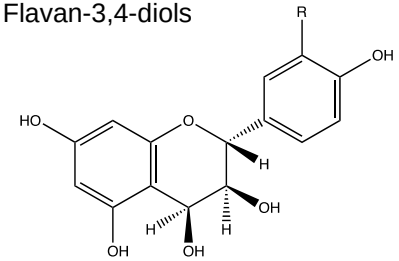
R=H, kaempferol
R=OH, quercetin

Flavan-3-ols



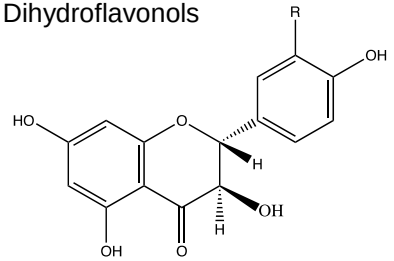
R=H, afzelechin
R=OH, catechin

Flavan-3,4-diols



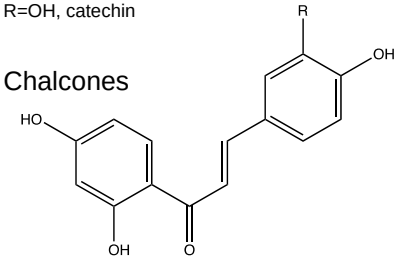
R=H, leucopelargonidin
R=OH, leucocyanidin

Dihydroflavonols



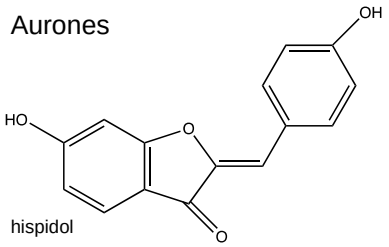
R=H, dihydrokaempferol
R=OH, dihydroquercetin

Chalcones



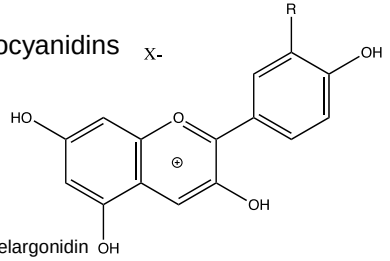
R=H, isoliquiritigenin
R=OH, butein

Aurones



hispidol

Anthocyanidins



R=H, pelargonidin
R=OH, cyanidin

2.2.1. Flavonoids

Flavonoids are compounds with low molecular weight and their structure is determined by a specific C_{15} system (C_6-C_3-C_6) of three rings, two of them (A, B) aromatic and one pyran type (C) (**Figure 4**) (22).

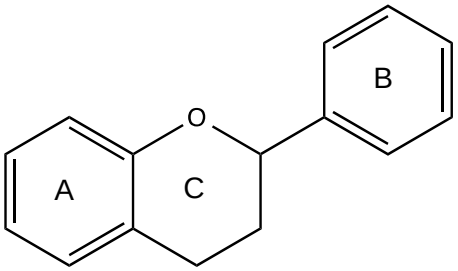


Figure 4. General structure of flavonoids.

These compounds are the most abundant group of phenolics and are divided into distinct subclasses according to the oxidation level of the central heterocyclic ring (**Table 2**).

The different stuctures of flavonoids come from various patterns of substitution through hydroxylation, methoxylation, sulfonation, acylation or glycosylation. The structural differences significantly affect their absorption, metabolism and bioactivities *in vivo* (21, 33).

There is marked evidence that diets rich in fruits and vegetables provide a reduced risk of chronic diseases. Flavonoids and related phenolic compounds that occur in plant-derived foods have been associated to these protective effects (21).

Anthocyanins are some of the most abundant flavonoids present in plants. The dark blue colour of *E. plantagineum* is a feature that distinguishes it from other pollen types. The anthocyanins profile of bee pollen loads presenting this dark blue/purple colour, like those from pollinic type *E. plantagineum,* has not been well studied (37).

2.2.2. Biosynthetic pathway

These compounds show great structural diversity, yet have some structural similarities because their biosynthetic origin derives from the aromatic amino acids phenylalanine and tyrosine (8).

The synthesis of aromatic compounds starts in plastids with the shikimic acid pathway and results in the production of three aromatic amino acids, tyrosine, phenylalanine and tryptophan (**Figures 5** and **6**). L-Phenylalanine occupies a key position as the precursor of the phenylpropanoid pathway. The cytoplasmic enzyme phenylalanine ammonia-lyase (PAL) removes the amino group from phenylalanine and yields *trans*-cinnamic acid, which is a substrate for a Cyt P450 monoxygenase. The product is *p*-coumaric acid, which can be further modified, usually by esterification with coenzyme A. The resulting pool of coumaroyl-CoA in the cytoplasm is the source of the synthesis of primary products, monolignols and lignin, and of a large number of natural products, the vital flavonoids and their derivatives and defensive compounds, such as coumarins (8).

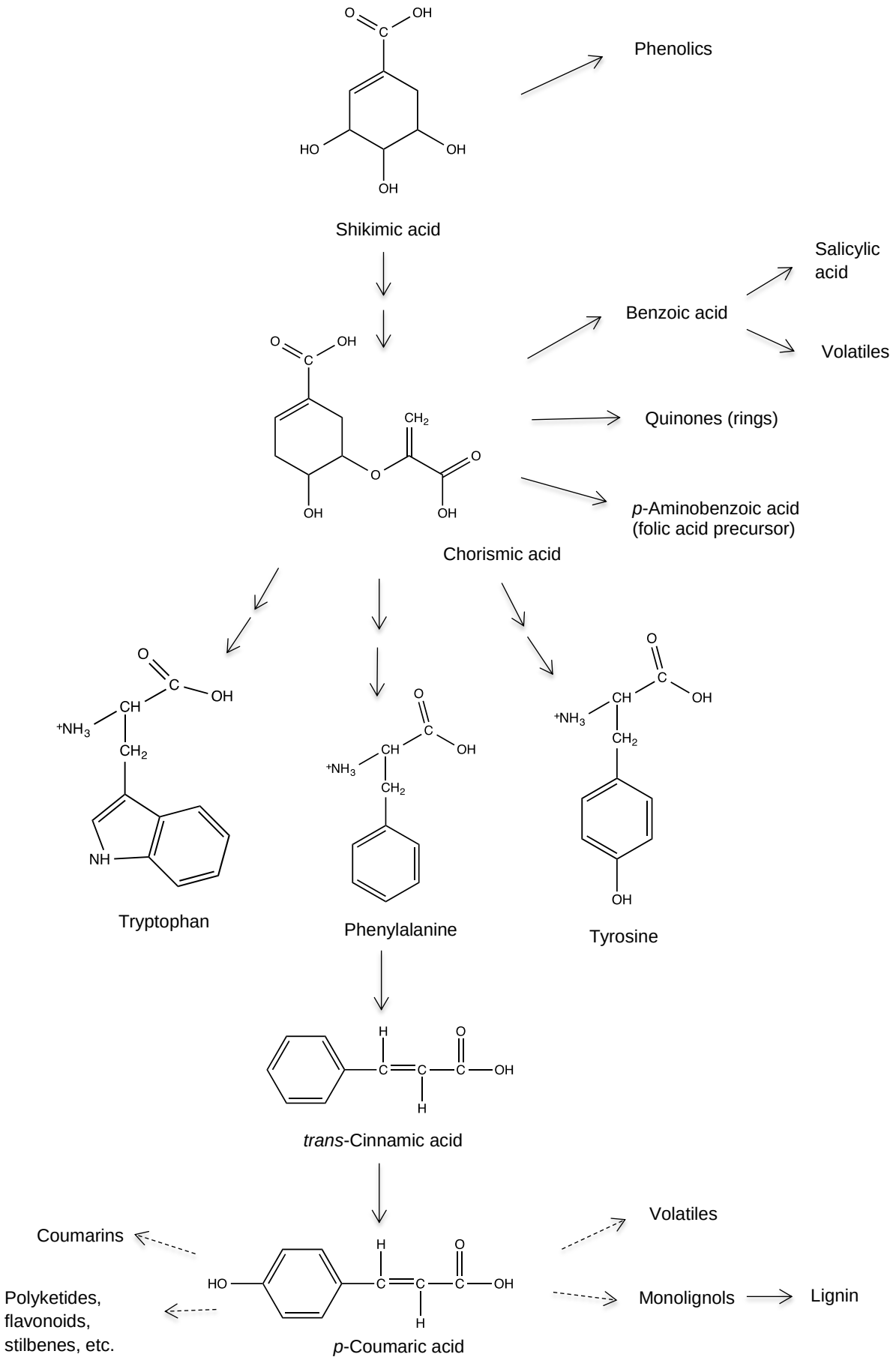


Figure 5. Shikimic acid pathway (simplified overview).

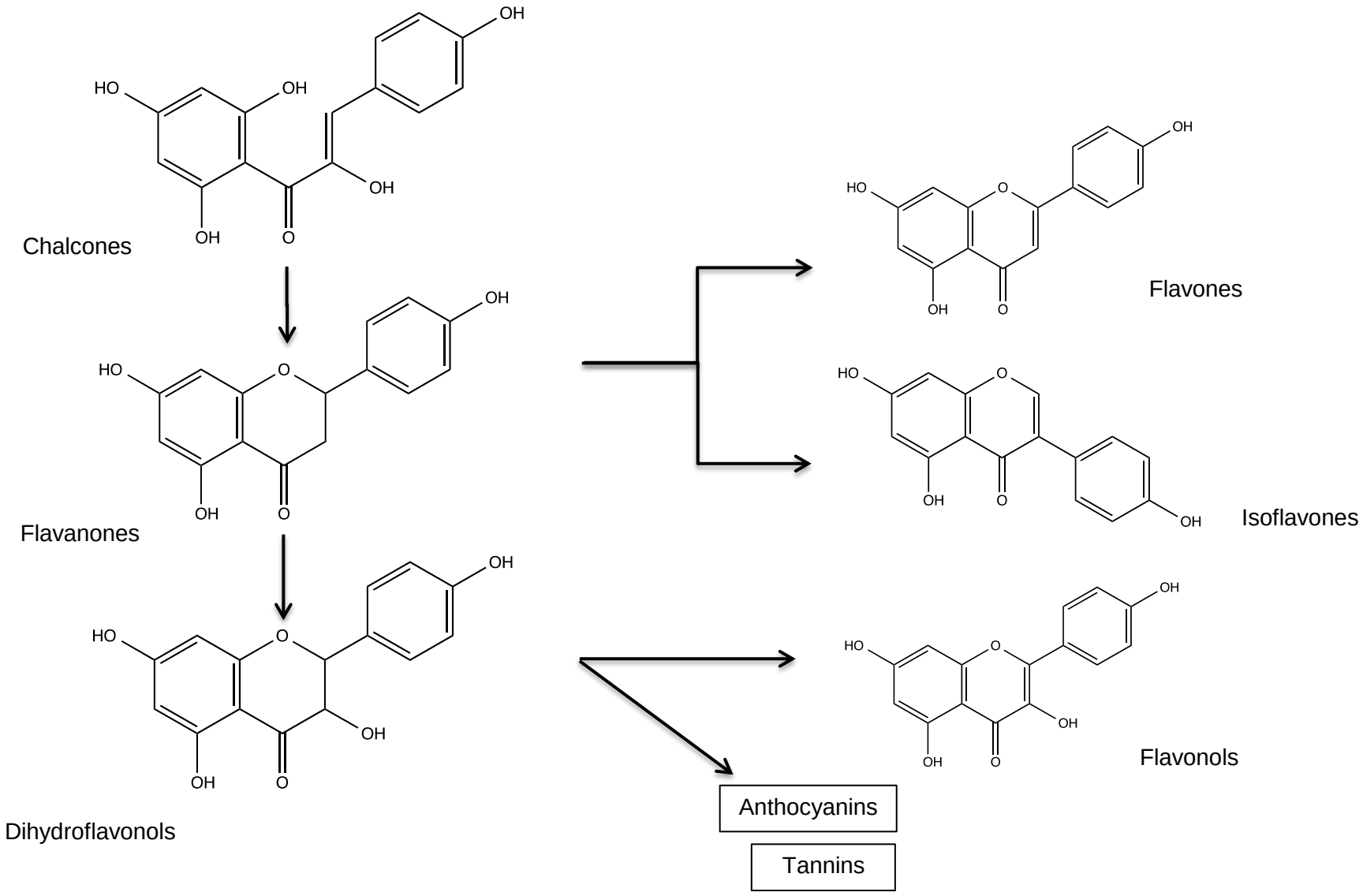


Figure 6. Simplified synthesis of flavonoids and tannins.

2.2.3. Extraction, separation and identification

The most important steps for the analysis of phenolic compounds are sample preparation and extraction, followed by identification and quantification using spectrophotometry, gas chromatography (GC), high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) methods (25).

The most common techniques to extract phenolic compounds employ solvents, either organic or inorganic. There are several parameters that may influence the yield of phenolics, including extraction time, temperature, solvent-to-sample ratio, the number of repeated extractions of the sample, as well as the solvent type. Besides, the optimum recovery of phenolics is different from one sample to another and relies on the type of plant and its active compounds. The choice of the extraction solvent, such as water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol), as well as of its mixtures, will influence the yield of phenolics extracted (25).

The association with ultrasounds enhances the process, with consequent reduction of the extraction time. Ultrasounds cause disruption of cell walls, reduction of particle size and enhancement of the mass transfer of cell contents to the solvent, by the collapse of the bubbles produced by cavitation, which leads to membrane's destruction (26, 27).

One of the disadvantages of solvent extraction is the co-extraction of other compounds, such as chlorophylls, peptides, proteins, sugars and organic acids, among others. To bias these interferences, different techniques can be performed, the use of C18 Sep-Pak cartridges being the most common (28).

Increasing time and temperature promote analyte solubility; however, plant phenolics are generally degraded or undergo undesirable reactions, like enzymatic oxidation by extended extraction times and high temperatures (25).

The increase of solvent-to-sample ratio enhances phenolic extraction from plant samples, but determining the optimum ratio is advisable, so that the solvent input and saturation effects of solvent by phenolics are minimized (25).

Sample matrix and particle size also strongly influence phenolics extraction from plant materials. Phenolic compounds may bind to other sample elements, such as carbohydrates

and proteins. These linkages can be hydrolysed by addition of enzymes, thereby promoting the release of bounded phenolics. Acidic and alkaline hydrolysis is also used in the isolation of phenolics from plants and plant products and is important for the stability of these compounds in the extract (25).

The purification stage includes removing interfering compounds from the crude extract, with partionable solvents and using open column chromatography or an adsorption-desorption process. Sephadex LH-20, polyamide, Amberlite, solid phase extraction (SPE) cartridges and styrene-divinylbenzene (XAD 4, XAD16, EXA-90, EXA 118, SP70), acrylic resins (XAD-7, EXA-31) are examples of regularly applied materials to purify phenolics from crude sample extracts. However, in most studies, SPE is used for purification and partial concentration prior to separation using HPLC (25).

Several techniques are used for the analysis of phenolics, namely thin-layer chromatography, gas chromatography, capillary electrophoresis, high-speed counter current chromatography, supercritical fluid chromatography, among others, reversed-phase HPLC being the preferred one for separation and quantification of phenolic compounds from plant-food material (25).

Various factors affect HPLC analysis of phenolics: sample purification, mobile phase, column type and detector (24). As referred below, HPLC equipment, provided with a reversed-phase C18 (RP-C18) column and diode array detector (DAD), and polar acidified organic solvents are most used to analyse purified phenolics. Usually, HPLC sensitivity and detection is based on purification of phenolic compounds and pre-concentration from complex matrices of crude plant extracts. The retention time of phenolic compounds is higher for substances that are less polar (flavonoids), while polar molecules (phenolic acids) are eluted more easily. However, if the compound contains apolar substituents, such as methoxyl groups, retention time increases; if it includes sugars or quinic acid residues it tends to elute before the corresponding free compound. So, retention time can be used as an indicator of polarity, allowing predicting the class of metabolite according to the chromatographic behavior (25).

The main eluents used in the HPLC analysis of phenolics are acetonitrile and methanol, or their aqueous mixtures. In some cases, ethanol, tetrahydrofuran (THF) and 2-propanol have been used. To avoid the ionization of phenolic and carboxylic groups during identification, it is necessary to maintain the pH of the mobile phase between 2 and 4, which will improve

both retention time and resolution. Therefore, aqueous acidified mobile phases predominantly contain acetic acid, but formic and phosphoric acids or phosphate, citrate and ammonium acetate buffers at low pH have also been used (25).

A gradient elution system is more commonly applied than an isocratic one (28), due to the chemical complexity and similarity of phenolic compounds.

The selection of the appropriate column is another critical factor in identifying phenolics. Based on the polarity, different classes of phenolics can be detected using silica-bonded C18 or RP-C18 column, 10-30 cm in length, 3.9-4.6 mm internal diameter and 3-10 μm particle size. Most HPLC analyses of phenolic compounds are carried out at room temperature. HPLC running time is the other factor that influences the detection of phenolics and can range from 10 to 150 minutes (25).

Phenolic compounds are frequently detected using UV-Vis and DAD detectors, at wavelengths ranging from 190 to 600 nm. DAD has the great advantage of supplying a wide range of information from a single run, as each class has a characteristic UV-Vis spectrum (27). Moreover, it gives the possibility to determine the correct wavelength to quantify the compounds and evaluate peak purity (30). Other methods also used are fluorimetric, colorimetric arrays, DAD coupled with fluorescence and chemical reaction-detection techniques (25).

HPLC coupled with mass spectrometry (MS) detector is highly sensitive and is able to achieve high specificity due to the mass selectivity of detection. Although UV-Vis information is a very important analytical tool, it may not be sufficient for the complete characterization of a complex mixture (25). Electrospray ionisation (ESI) is the method better suited to couple HPLC with MS (31) when the purpose is to analyse thermally unstable compounds with higher molecular weights and polarity. As this technique has a good sensitivity for flavonoids glycosides present in plant extracts, it is one of the most widely used (28).

2.2.4. Biological activities

The growing interest in phenolic compounds, especially flavonoids, is mainly due to their antioxidant potential and the association between their consumption in fruits and vegetables and the prevention of some diseases, mainly those associated to oxidative stress, like degenerative ones (cancer, multiple sclerosis, autoimmune, Alzheimer's and Parkinson's diseases) (30, 36). Active oxygen free radicals are involved in oxidative stress, being

implicated as causative agents in the conditions metioned above (36). These species can be produced both by normal metabolism and by external influences, like UV light and carcinogens. When produced in quantities that overload the body's natural antioxidant and repair defence system, they can bring about breakdown of vital components, such as coenzymes, neurotransmitters and macromolecules (e.g., nucleic acids, proteins, lipids and carbohydrates) (36). Living cells have a limited capacity to nullify the activity of these oxidative free radicals. The regular intake of phenolic compounds can improve the protection of vital cellular components and, thus, their physiological function, bringing health benefits to the individual (36).

The antioxidant properties of phenolic compounds depend on the chemical structure, nucleus rearrangement and the presence of functional groups (32, 33). Phenolic compounds have an aromatic ring bearing one or more hydroxyl groups and their structure may vary from that of a simple phenolic molecule to that of a complex high molecular mass polymer. In the presence of free radicals phenolics have the capacity to stabilize the unpaired electron, by donating a hydrogen atom of their hydroxyl groups, and stabilize the phenoxyl radical so formed, converting it into an innocuous molecule (30, 32).

Quercetin (3,3',4',5,7-pentahydroxylflavone) is a flavonol-type flavonoid ubiquitously present in vegetables (**Figure 7**). This compound has high antioxidant capacity, directly scavenging reactive oxygen species (ROS) or effectively inhibiting ROS-generating enzymes (e.g. xanthine oxidase, lipoxygenase) (34). This is mainly due to the presence of the two hydroxyl groups in ring B. In addition, the presence of a 3-OH group increases the antioxidant activity, since it turns planar the conformation of ring B, allowing conjugation to occur and an electronic stability displacement reaction (32). Also, the double bond between C2 and C3 influences electronic displacements and maintains the planar conformation. This explains why flavonols and flavan-3-ols are generally the classes of flavonoids with stronger antioxidant activity (32).

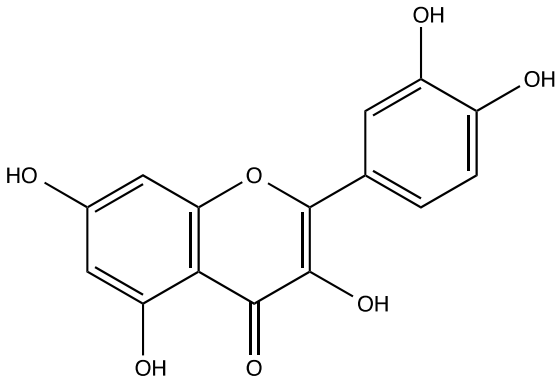


Figure 7. Quercetin (3,3',4',5,7-pentahydroxylflavone).

Hydroxycinnamic acids have some structural features similar to those of flavonoids (aromatic ring bearing one or more hydroxyl groups, double bond, carbonyl group) that may be important for their antioxidant capacity. Furthermore, phenolic compounds are also known to increase cell levels of antioxidant enzymes, like superoxide dismutase, glutathione peroxidase and catalase, and to inhibit the pro-oxidant ones, such as xanthine oxidase and lipoxygenases (35).

2.2.5. Phenolic compounds in *E. plantagineum*

Several coloured and non-coloured flavonoids were described in *E. plantagineum* pollen:

- Coloured flavonoids (anthocyanins) - delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, petunidin-3-*O*-glucoside, petunidin-3-*O*-rutinoside, peonidin-3-*O*-rutinoside, malvidin-3-*O*-rutinoside, cyanidin-3-(6"-malonylglucoside) (37). The anthocyanin present in highest amount is petunidin-3-*O*-rutinoside, accounting for 80 to 90% (37) (**Figure 8A**).

- Non-coloured flavonoids - quercetin-3-*O*-sophoroside, isorhamnetin-3-*O*-rutinoside, quercetin-3-*O*-neohesperoside, kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-(4"-rhamnosyl)-neohesperidoside, kaempferol-3-*O*-neohesperidoside derivative, kaempferol-3-*O*-(3"/4"-acetyl)-neohesperidoside, kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-(4"/3"-acetyl)-neohesperidoside isomer (14). Of these, the most abundant determined flavonoid is kaempferol-3-*O*-neohesperidoside (14) (**Figure 8B**).

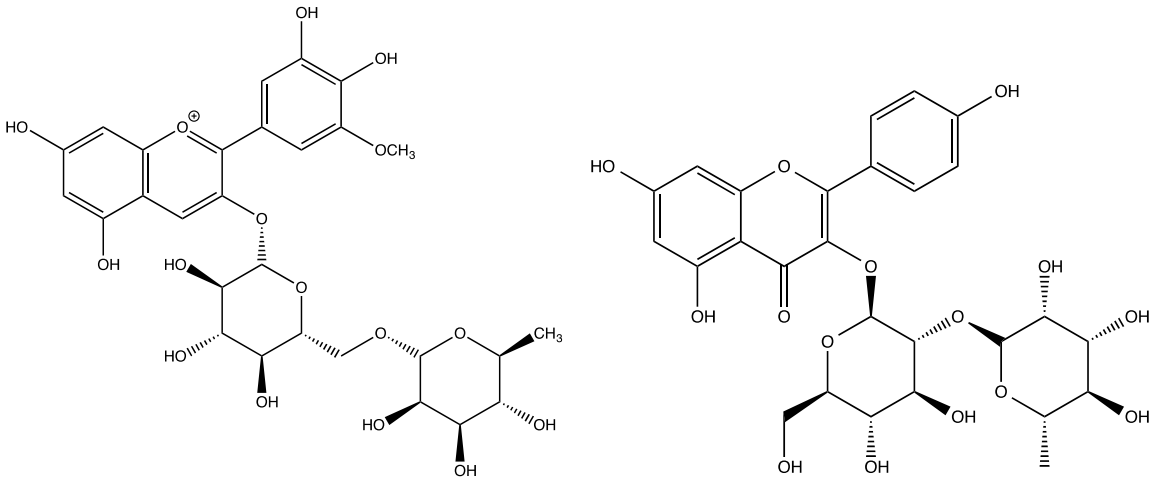


Figure 8. Chemical structures of petunidin-3-O-rutinoside (A) and kaempferol-3-O-neohesperidoside (B).

3. Primary metabolism

3.1. Organic acids

Organic acids are naturally occurring compounds present in foods of plant origin, as well as produced during fermentation of foods. Organic acids metabolism is involved in several cellular biochemical pathways, including energy production, formation of precursors for aminoacids biosynthesis and, at the whole plant level, in modulating adaptation to the environment (40).

The high accumulation of organic acids in plant tissues is most probably due to their important role as photosynthetic intermediates. However, organic acids have a potential role as metabolically active solutes for the osmotic adjustment and the balance of cation excess. Organic acids also participate as key components in the mechanisms that some plants use to cope with nutrient deficiencies, metal tolerance and plant–microbe interactions, operating at the root–soil interface (38).

3.1.1. Biosynthetic pathway

Organic acids are mainly produced in mitochondria through the tricarboxylic acids or Krebs cycle and, to a lesser extent, in the glyoxysome as part of the glyoxylate cycle (**Figure 9**). Because of the catalytic nature of the Krebs cycle, organic acids are present only in very small pools in the mitochondria and are preferentially stored in the vacuole (40). The most common organic acids are the carboxylic acids, whose acidity is related with their carboxyl group. Malonic and acetic acids are precursors of fatty acids and also of flavonoids (together with shikimic acid) (40).

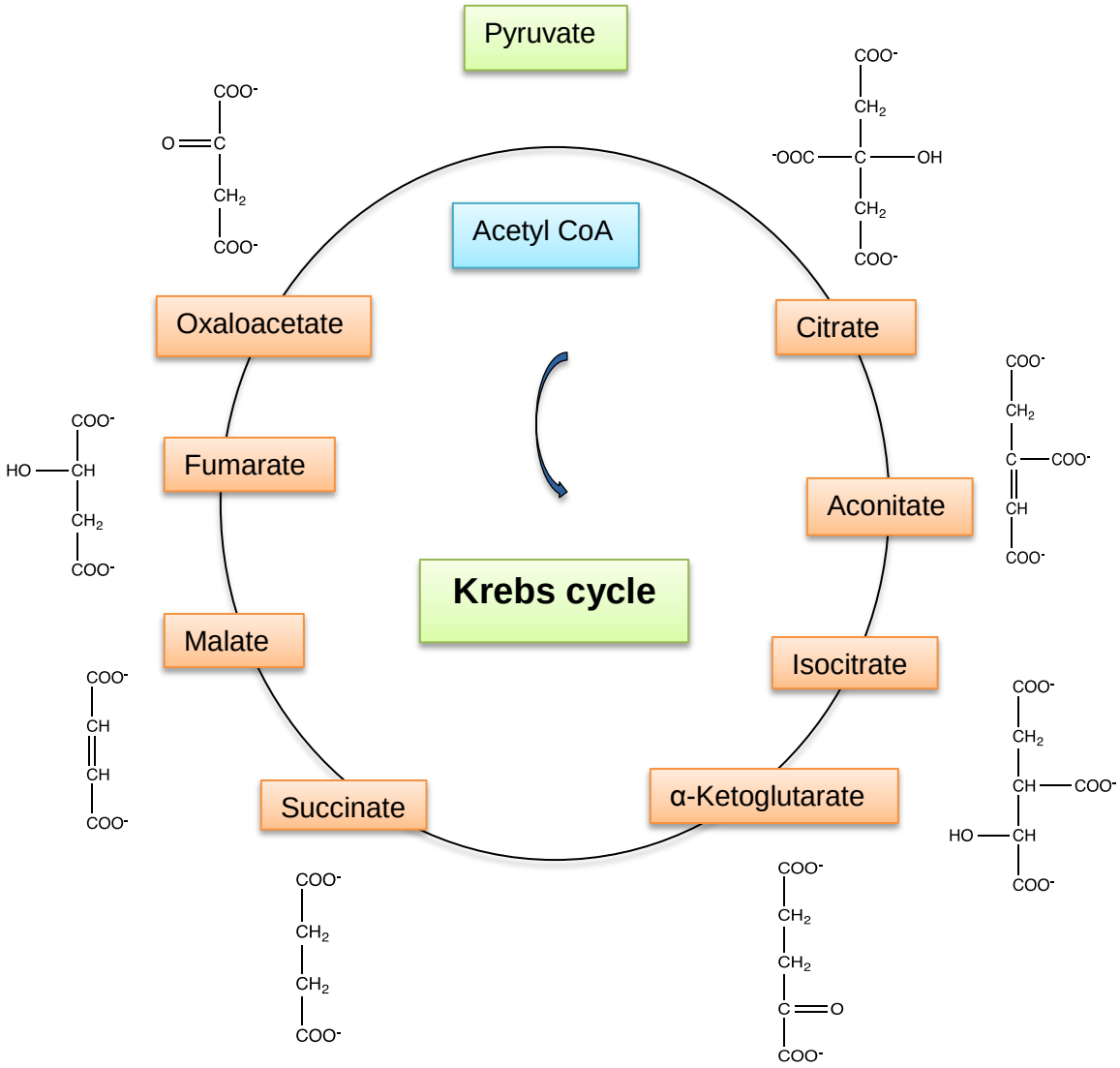


Figure 9. Krebs cycle.

3.1.2. Extraction, separation and identification

Organic acids are soluble in water and hydroalcoholic solvent mixtures. The extraction of organic acids is increased when the solvent is acidified and further purification of the extracts is achieved using SPE (41, 42).

HPLC is a particularly useful method to separate polar or moderately polar compounds, such as organic acids (43). An ion exclusion column, consisting on an ion-exchange resin, splits ionised and neutral species. While ionised compounds are rejected by the resin and eluted through the column, the neutral and slightly ionised ones are retained. This allows proper separation of acid molecules from highly ionised compounds (44). Elution can be isocratic or in gradient mode, the first being more used for the determination of organic compounds (45, 46).

The detection of organic acids can be achieved by several techniques, such as UV, MS and fluorescence (43). The most commonly used is UV, in which case external standards for compounds' identification and quantification are necessary (41).

3.1.3. Biological activities

Organic acids have long been utilized as food additives and preservatives for preventing food deterioration and extending the shelf-life of perishable food ingredients (38). Although the mechanisms of organic acids antibacterial effect are not fully understood, they are capable of exhibiting bacteriostatic and bactericidal properties, depending on the physiological status of the organism and the physiochemical characteristics of the external environment. Given the weak acid nature of most of these compounds, pH is considered a primary determinant of effectiveness, because it affects the concentration of undissociated acid form. It has been traditionally assumed that undissociated forms of organic acids can easily penetrate the lipid membrane of the bacterial cell and once internalized into the neutral pH of the cell cytoplasm dissociate into anions and protons (38, 39). The generation of these species potentially presents problems for bacteria that must maintain a neutral cytoplasm to sustain macromolecules. The exportation of excess of protons requires consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy (38, 39). Other toxicity mechanisms have been proposed for organic acids, like membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis and the accumulation of toxic anions (39, 47, 48).

They can be interesting to prevent several diseases like cancer and atherosclerosis (47). At industrial level they can be used as flavourings, preservatives and antioxidants. The utilization of organic acids is expected to increase because of the increasing demand for organic foods (48).

3.2. Fatty acids

Fatty acids can be represented by the general formula RCOOH, in which R corresponds to an alkyl chain. Long-chain fatty acids with an even number of carbon atoms are important for human nutrition and metabolism (12 to 26). These metabolites can be classified according to the degree of saturation. Saturated fatty acids contain a flexible alkyl chain, without any double bond between carbon atoms; the monounsaturated have a double bond and the polyunsaturated have at least two of those bindings. There are *cis* or *trans* isomers (48).

As the fatty acid carboxylic group has a pKa near 4.8, these molecules are found in body fluids in the ionized form. Humans are able to synthesize the majority of the fatty acids, including saturated, monounsaturated and some polyunsaturated ones. However, linoleic and α-linolenic acids (**Figure 10**) are exceptions, being considered essential fatty acids (49).

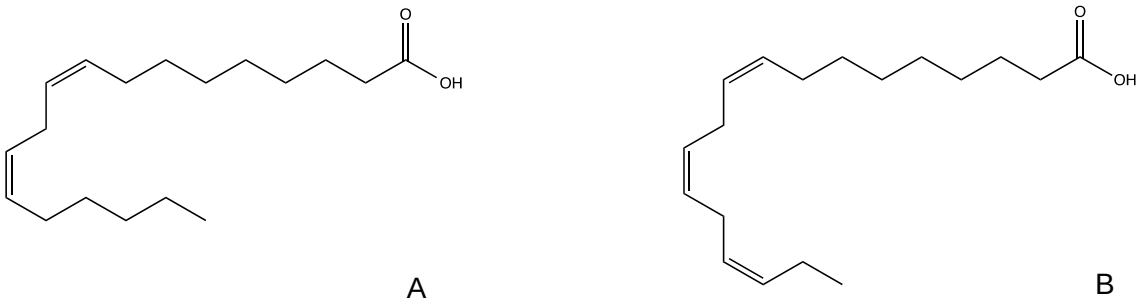


Figure 10. Chemical structure of (A) linoleic acid (B) α-linolenic acid.

3.2.1. Biosynthetic pathway

Echium oil is a vegetable oil extracted from the seeds of *E. plantagineum* containing notable amounts of both ω-6 and ω-3 PUFA, *y*-linolenic acid (GLA) and stearidonic acid (SDA), respectively. Both SDA and GLA are the immediate products of the rate-limiting Δ6-desaturase step and, due to the efficiency of the elongase and Δ5-desaturase-mediated steps, are readily converted to long chain PUFAs (**Figure 11**) (50, 51).

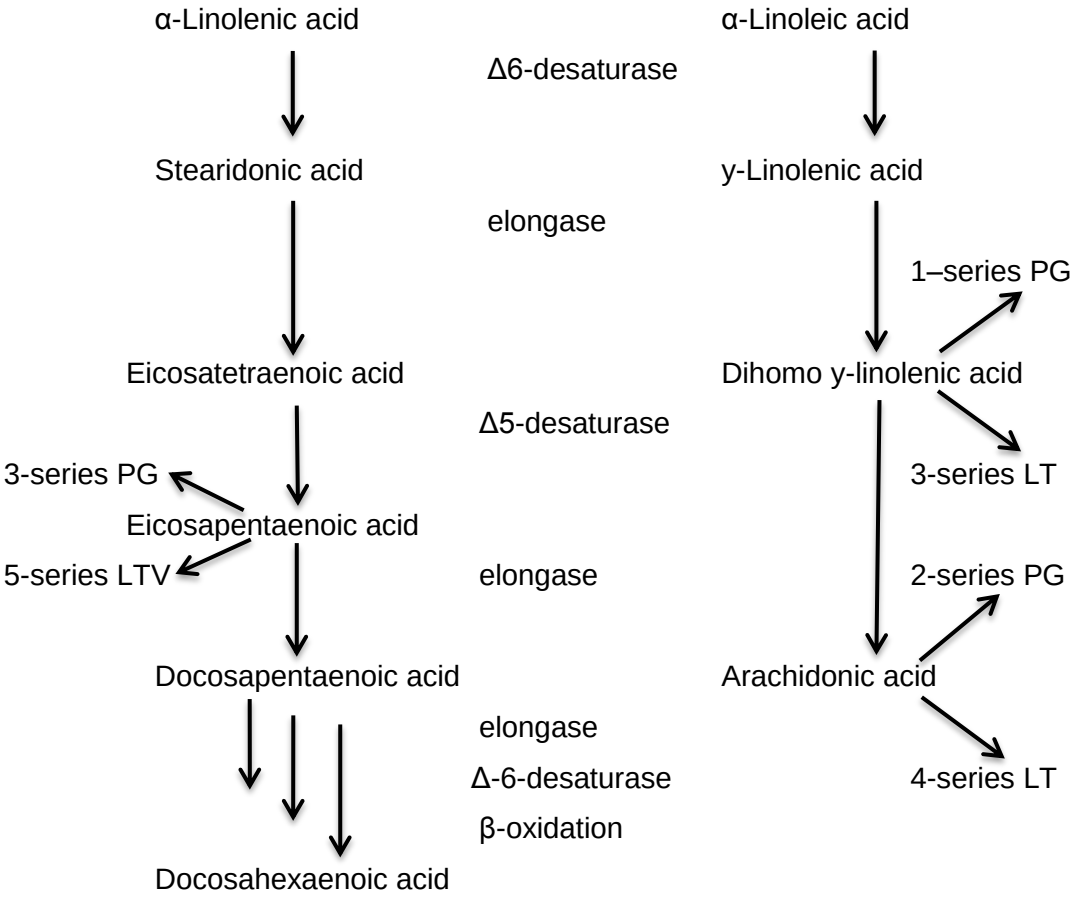


Figure 11. Metabolic pathway of ω-3 and ω-6 polyunsaturated fatty acids and derived eicosanoids [adapted from (51)]. LT: leukotriens; LTV: leukotriens series 5; PG: prostaglandins.

3.2.2. Extraction, separation and identification

The solvents used for fatty acids extraction should offer a good polarity range to extract both the polar lipids from cellular membranes and the non-polar lipids, a mixture of chloroform and methanol (2:1) being the most used (52).

The separation of fatty acids is mainly performed by GC-MS. In this context, the samples must be derivatized to give rise to less polar and sufficiently volatile compounds, which could be eluted at reasonable temperatures without undergoing thermal decomposition or molecular rearrangements (53).

Different methods, like esterification, silylation and acetylation, can be used for derivatization. Esterification is the most commonly used and can be done with an appropriate alcohol, such as methanol, and an inorganic acid to catalyse the reaction. Boron trifluoride or boron trichloride are very useful for forming ester derivatives, the reactions catalysed by the first being faster (53).

3.2.3. Biological activities

Lipids are an important component of the diet. There is a growing awareness that diet in general, and lipids in particular, are important in the maintenance of good health and in the development and treatment of some diseases. Fatty acids and their triacylglycerols represent a wide range of structures, and it is the total amount of these and the balance between the various types that it is significant (54).

The effects of fatty acids are complex and greatly vary according to the dose and the nature of the molecule. The knowledge on the beneficial effect of ω-3 long-chain PUFAs on inflammatory and autoimmune diseases, like atherosclerosis, cancer, rheumatoid arthritis, asthma, Alzheimer's disease and others, has dramatically increased during recent years (55). The literature also refers the fatty acids importance in the bone matrix, since they interfere with signalling pathways of osteoclasts and osteoblasts, favouring bone growth (56).

Among PUFAs, the ones belonging to ω-3 and ω-6 families are highlighted. The human organism is not able to synthesize them due to the absence of specific enzymes. So, it is necessary to obtain them from the diet (57, 58). These fatty acids are recognized by their ability to lower serum cholesterol levels, leading to the reduction of blood pressure, thus decreasing the risk of cardiovascular disease (59).

3.2.4. Fatty acids in *E. plantagineum*

It is known that pollen contains all nutrients, including lipids, which are necessary for plant growth and development. As far as we know, fatty acid content of *E*. *plantagineum* bee pollen has not determined. Nevertheless, the fatty acid composition of other tissues is well studied.

Echium plantagineum seed contains a highly polyunsaturated oil: 16% oleic acid (OA, 18:1 n-9), 19% linoleic acid (LA, 18:2 n-6), 10% γ-linolenic acid (GLA, 18:3 n-6), 30% α-linolenic acid (ALA, 18:3 n-3) and 13% stearidonic acid (SDA, 18:4 n-3). By their metabolism, this natural ratio of fatty acids delivers enhanced plasma concentrations of eicosapentaenoic

(EPA, 20:5 n-3), docosapentaenoic (DPA, 22:5 n-3) and dihomo-γ-linolenic (DGLA, 20:3 n-6) acids, without increasing the concentrations of arachidonic acid (AA, 20:4 n-6) (51). Almost all of the fatty acids belong to ω-3 family (60).

The main fatty acid in *E. plantagineum* leaf is ALA (61). Macaronesian *E. plantagineum* leaves have a high content of the following saponifiable lipids: 16:0 (20.26%) and 18:3 n-3 (29.43%) (61).

4. Inflammation

Inflammation is a physiological response induced by microbial infection or damage to living tissues. A major trigger of inflammation is the recognition of microbes by specific receptors of the innate immune system, which play a crucial role in the induction of early signals initiating and establishing the inflammatory setting (62). It is a defence mechanism that evolved in higher organisms to protect them from infection and injury. A main function of inflammation is to resolve infection and to repair damage in order to achieve homeostasis equilibrium (62). Thus, the ideal inflammatory response is rapid and destructive, yet specific and self-limiting. This is a process involved in the so-called acute inflammation because it lasts only a few days, allowing the aggressive agent to be localized and eliminated and the organism to heal. A response of longer duration is referred to as chronic inflammation, when chronic infectious or inflammatory disorders cause more damage to the host than the microbe (62).

Inflammation and the immune system are intimately related. So, an over activation of innate immune response can cause chronic infection or chronic inflammation, and hence disease, due to an inefficient regulation or resolution of the inflammatory response (62).

Oxidative stress is implicated in the inflammatory processes at the molecular level. Oxidative stress is a status characterized by the production of high amount of oxidants or low level of antioxidants, which results in an imbalance between oxidant and antioxidant systems causing damage. Oxidative stress can be extrinsically induced by environmental factors and intrinsically by endogenous factors, such as the electron transport chain in mitochondria, some enzyme activities (NADH oxidase and nitric oxide synthase) and respiratory burst from inflammatory cells (63).

Inflammation induces reactive oxygen species and reactive nitrogen species production *via* respiratory bursts and inflammatory cytokines, which can activate many oxidant generating enzymes, such as inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), myeloperoxidase (MPO) and eosinophil peroxidase (EPO) (63).

Eicosanoids, including prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs), are signalling molecules that are generated primarily through an oxidative pathway from AA (**Figure 12**). They are also generated from pathways originating from EPA and DGLA (64). AA-derived eicosanoids exert complex control over a wide range of physiological processes. Eicosanoids production is considerably increased during inflammation and their biosynthetic pathways are of particular clinical relevance because their products are involved in the pathogenesis of various pathologies related to immune functions (64).

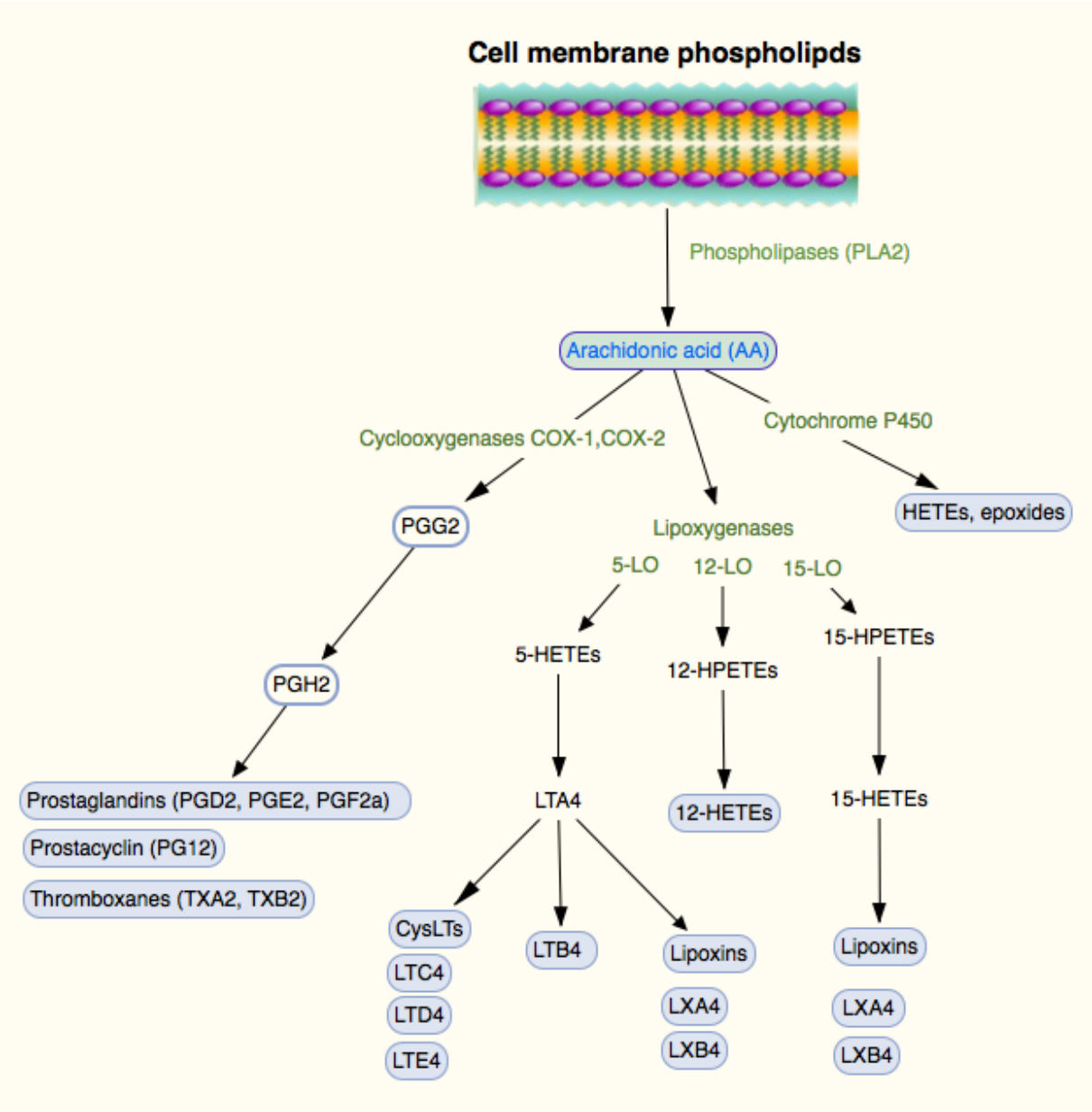


Figure 12. Synthesis of eicosanoids through an oxidative pathway from arachidonic acid (AA). Non-specific activating stimuli, including cytokines, hormones and stress, lead to AA release from membrane phospholipids by phospholipases, particularly cytosolic phospholipase A2 (cPLA2). Free AA can be converted to bioactive eicosanoids through the cyclooxygenase (COX), lipoxygenase (LOX) or Cyt P450 epoxygenase pathways. LOXs catalyse the formation of LTs, 12(S)hydroperoxyeicosatetraenoic acids and lipoxins (LXs). COX isozymes (constitutive COX-1 and inducible COX-2) catalyse the formation of PGH2, which is converted by cell-specific PG synthases to biologically active products, including PGE2, PGF2α, PGI2 and TXA2, collectively known as prostanoids. The Cyt P450 epoxygenase pathway catalyses the formation of hydroxyeicosatetraenoic acids (HETEs) and epoxides [from (64)].

The biosynthesis of eicosanoids depends on the availability of free AA. When tissues are exposed to diverse physiological and pathological stimuli, such as growth factors, hormones or cytokines, AA is produced from membrane phospholipids by the action of phospholipase

A2 (PLA2) enzymes and can then be converted into different eicosanoids. AA can be enzymatically metabolized by three main pathways: Cyt P450 epoxygenase, cyclooxygenases (COXs) and lipoxygenases (LOXs) (**Figure 12**). The Cyt P450 epoxygenase pathway produces hydroxyeicosatetraenoic acids (HETEs) and epoxides. The COX pathway produces PGG2 and PGH2, which are subsequently converted into PGs and thromboxanes (TXs).

There are two COX isoforms, commonly referred to as COX-1 and COX-2 (65). It is the enzyme that catalyses the rate-limiting step in prostaglandin synthesis, converting AA into prostaglandin H2α, which is then further metabolized to prostaglandin E2 (PGE2), PGF2α, PGD2 and other eicosanoids.

COX-1 is constitutively expressed in many tissues (encoded by a constitutively expressed gene) and plays an important role in tissue homeostasis. COX-2, which can be expressed in a variety of cells and tissues, is an inducible isoform (encoded by an immediate early response gene) and growth factors, cytokines and tumour promoters stimulate its expression (66). Despite the structural similarity between the two isoforms, COX-1 and COX-2 differ substantially in the regulation of their expression and their roles in tissue biology and disease.

LOXs are more numerous and convert AA into diverse hydroperoxyeicosatetraenoic acids (HPETEs) and HETEs. 5-HETE is converted into the leukotriene LTA4, which is the precursor of LTB4, cysteinyl-LTs (CysLTs) (including LTC4, LTD4 and LTE4) and LXs. Synthesis of LXs is dependent on the activity of the requisite interacting LOXs and the proximity of cells that are necessary for the metabolism of AA to the LX end-products. In some instances, the metabolite is transferred to another cell that, in turn, converts it into another compound. For example, PGI2 and LXA4 can be produced during cell–cell interactions, utilizing enzymes in adjacent cells. PGI2 is produced from PGH2 (of platelet origin) by the vascular epithelium or lymphocytes. Similarly, LTA4 produced by neutrophils can be converted into LTC4 by vascular epithelium or platelets, or into LTB4 by erythrocytes. Thus, biosynthesis of different eicosanoids is dependent on local production and distribution of specific precursors and enzymes in specific cells (64).

4.1. *In vitro* models

Macrophages are a major component of the mononuclear phagocyte system that consists

of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages. They play a critical role in the initiation, maintenance, and resolution of inflammation, being activated and deactivated in the inflammatory process. Activation signals include cytokines (interferon gamma, granulocyte-monocyte colony stimulating factor, and tumor necrosis factor alpha (TNF-α)), bacterial lipopolysaccharide (LPS), extracellular matrix proteins, and other chemical mediators. Inhibition of inflammation by removal or deactivation of mediators and inflammatory effector cells permits the host to repair damaged tissues. Anti-inflammatory cytokines (interleukin 10 and transforming growth factor beta) and cytokine antagonists that are mainly produced by macrophages deactivate activated macrophages. Macrophages participate in the auto-regulatory loop in the inflammatory process. Because macrophages produce a wide range of biologically active molecules participating in both beneficial and detrimental outcomes in inflammation, therapeutic interventions targeting macrophages and their products may open new avenues for controlling inflammatory diseases (67).

The cellular model of LPS-stimulated macrophages is widely used to assess anti-inflammatory activity (68, 69, 70). It is well known that infection of cells by microorganisms activates the inflammatory response due to the recognition by macrophages receptors of LPS present in microorganisms' cell wall: the initial sensing of infection is mediated by innate pattern recognition receptors that are expressed by macrophages dendritic cells and various nonprofessional immune cells. Although inflammation is a protective response of the body to ensure removal of detrimental stimuli, as well as a healing process for repairing damaged tissue, the stimulation of macrophages, initiated by microorganisms (or other agent) can result in the overproduction of inflammatory mediators like PGE2 and PGI2, which enhance edema formation and leukocyte infiltration by promoting blood flow in the inflamed region (71, 72).

5. Allergy

Bee pollen has been used as a "perfect health food" for many centuries and its benefits have been widely lauded. Modern research has also shown that bee pollen mainly possesses the therapeutic effects of improving the cardiovascular system, enhancing body immunity, delaying aging, maintaining the digestive system and preventing prostate degeneration (73). Nevertheless, in sensitized individuals it can cause allergic reactions.

Allergies are immune-mediated hypersensitivity reactions that can affect various organs, most commonly the skin, airways and gastro-intestinal tract (74). The route of exposure, dose and function of the allergen are crucial to mount an allergic sensitization (74). There is ample evidence that allergic disorders, such as asthma, rhinitis, and atopic dermatitis, are mediated by oxidative stress (75).

Allergy is mainly classified into four types, type I being the most common allergic reaction associated with asthma and allergic dermatitis. The type I allergic reaction is provoked by cross-linkage of an antigen, referred to as allergen to immunoglobulin E (IgE) bound on the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. The cross-linked FcεRI activates the Src family non-receptor tyrosine kinases Lyn and Fyn. Activation of Lyn induces phosphorylation of the Syk kinase that leads to Ca^{2+} mobilisation. As a result, chemical mediators like histamine, eicosanoids and inflammatory cytokines are released from intracellular granules (76). Released histamine induces an acute allergic response, such as contraction of smooth muscle, vasodilation and increased vascular permeability (**Figure 13**) (76).

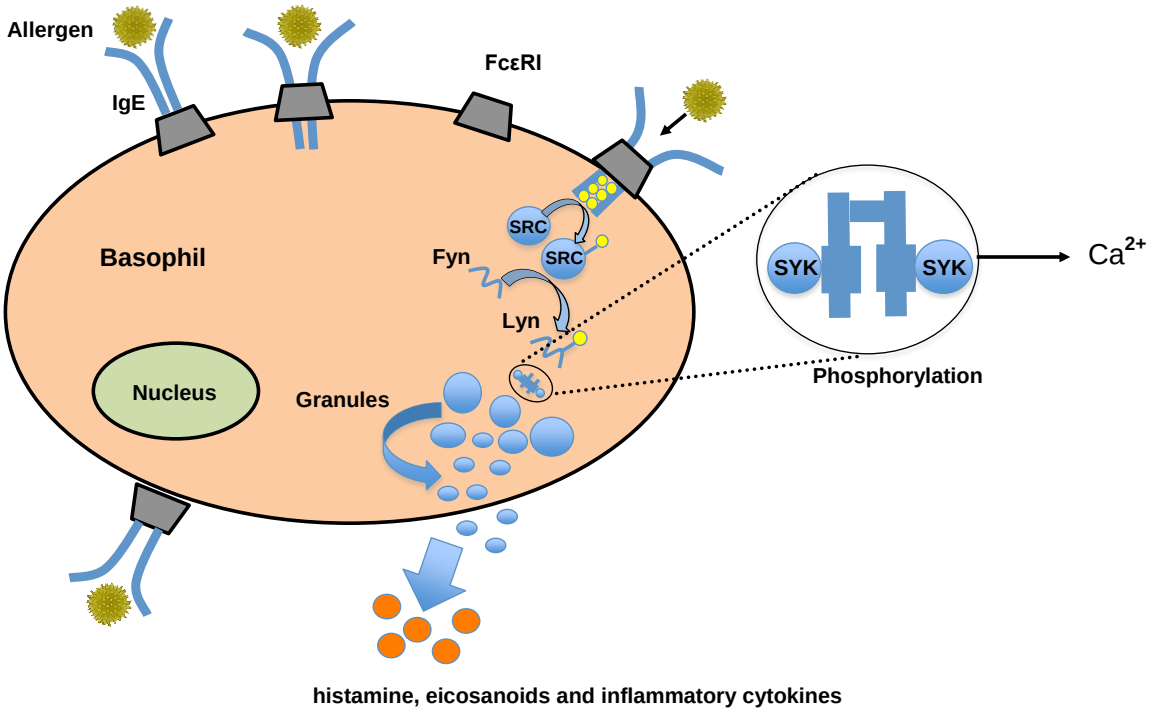


Figure 13. Type I allergic disease mechanism.

The most important sources of allergens are wind-dispersed pollen grains from trees, grasses and weeds, followed by excretions of house dust mites and cockroaches, fungal spores, animal dander and insect venoms (74).

Notwithstanding the genetic predisposition, there are several factors that contribute to the appearance of allergic disease, which can be classified as adjuvant and causal factors. As adjuvant factors that cause allergic sensitisation, we can mention biogenic and antropogenic factors; irritants, environmental tobacco smoke, infections and exercise cause skin and/or airway hyper-reactivity (74). As causal factors, allergens are the main ones promoting allergic sensitisation; oligomerisation and surface motifs are responsible for skin and/or airway hyperreactivity (74).

Atopic diseases are characterized by predominance of T helper cell type 2 (Th2)-biased immune responses to environmental allergens (77, 78). It is well established that allergen specific Th2 cells are the key orchestrators of allergic reactions, initiating and propagating inflammation through the release of a number of Th2 cytokines (78). Th2 responses constitute a prerequisite for the development of type I hypersensitivity reactions, which lead to IgE production and arming of mast cells and basophils with specific IgE. Subsequent allergen encounter leads to IgE-mediated mast cell/basophil degranulation and release of pro-inflammatory mediators, cytokines, and chemokines that ultimately are responsible for the inflammatory response seen in type I hypersensitivity (75) (**Figure 14**).

In the context of type I allergy, airborne pollen grains have generally been regarded as allergen carriers. Aside from allergens, pollens release many other substances with pro-inflammatory and immune-modulatory effects on cells of the allergic immune response. In addition, pollens can carry biogenic and anthropogenic factors that influence allergen release, generate novel allergenic epitopes, and modulate the epithelial microenvironment of an allergen encounter (74).

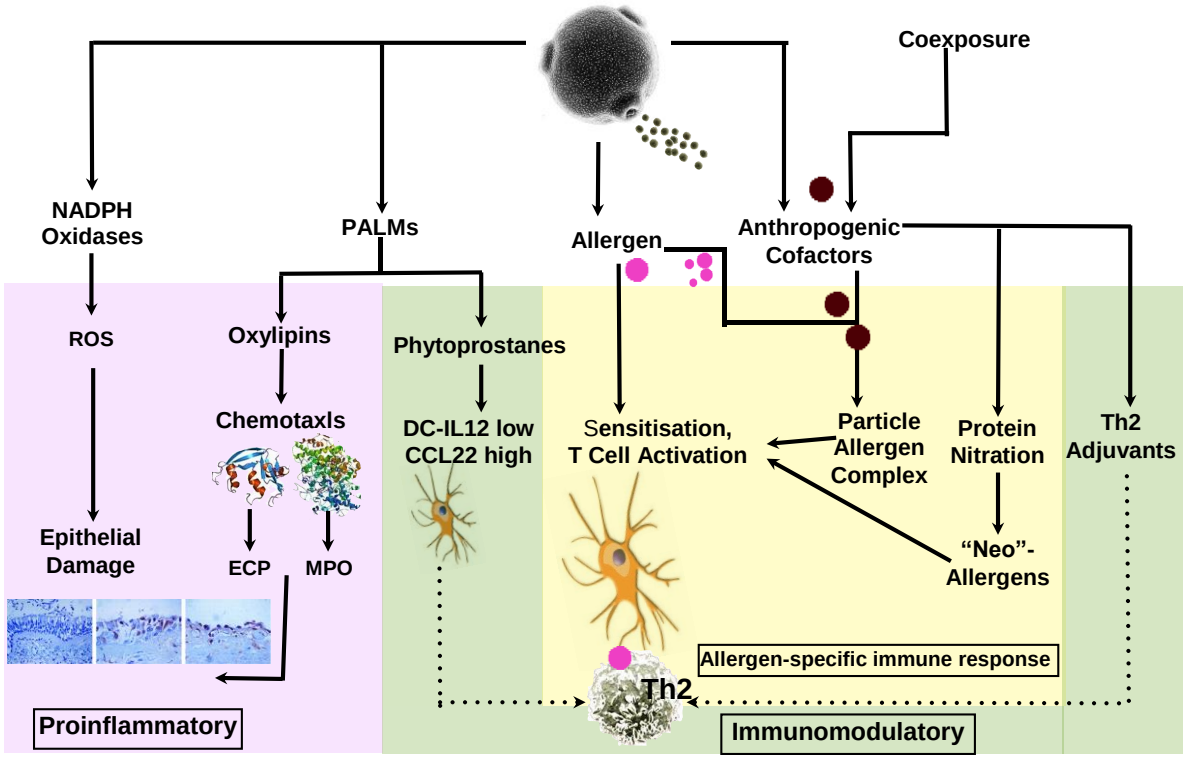


Figure 14. Interplay between the allergen source, environmental cofactors and cells of the allergic immune response during exposure to pollen grains. NADPH, Reduced nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; ECP, eosinophil cationic protein; MPO, myeloperoxidase; PALMs, pollen-associated lipid mediators [adapted from (74)].

In fact, it has been demonstrated that pollen grains secrete significant amounts of lipids that are structurally and functionally homologous to eicosanoids (78): pollen grains not only release allergens, but also non-allergic, bioactive and pollen-associated lipid mediators (PALMs), which have pro-inflammatory and immune-modulatory effects on the cells of the allergic immune response. Pro-inflammatory PALMs (eg. oxylipins) attract and activate eosinophils and neutrophils independently of the sensitization status of the donor (i.e., from allergic and non-allergic donors), suggesting that they act primarily as an adjuvant that can enhance inflammatory processes, such as during the elicitation of the allergic response (74). Immuno-modulatory PALMs like E1 phytoprostanes inhibit dendritic cells (DC) production of IL-12 and Th1-type chemokines and increase the capacity of DCs to induce Th2 cell differentiation and recruitment, which indicates that they support the generation of a Th2 cell-dominated allergic immune response (74). Finally, pollen releases non-allergic nicotinamide adenine dinucleotide phosphate oxidases, which have detrimental effects on airway epithelial cells through the generation of reactive oxygen species (74).

5.1. *In vitro* models

During immediate-type allergy (type I hypersensitivity), histamine is released from activated mast cells *via* a degranulation process by IgE-allergenic stimulation. Along with histamine release β-hexosaminidase is simultaneously degranulated from the same activated cells (79). Thus, β-hexosaminidase activity can be used as a biomarker of allergic response of the mast cells. As indicated above, 5-lipoxygenase (5-LOX) is an enzyme that produces LTs from AA. Because LTs are mediators of some allergic reactions, such as bronchial asthma (80), 5-LOX is regarded as an enzyme that induces allergic response. Accordingly, inhibition of the release of β-hexosaminidase and 5-LOX is well correlated with anti-allergic action. On the other hand, the recruited monocytes and lymphocytes and their secreted cytokines like IL-1β play an important role in delayed-type allergic response (type IV hypersensitivity) (81).

The rat basophilic leukemic (RBL-2H3) cell line has been widely used for allergy and immunological research (82, 83, 84). The release of β-hexosaminidase enzyme, a marker of degranulation in mast cells and basophils, allows estimating the anti-allergic potential of new drugs *in vitro* (85).

6. Objectives

The main objectives of this dissertation were:

1. To improve the knowledge on the chemical composition of *E. plantagineum* bee pollen;

2. To evaluate the potential of *E. plantagineum* bee pollen on inflammation and to elucidate the mechanisms underlying the anti-inflammatory effect;

3. To evaluate the activity of *E. plantagineum* bee pollen on allergy and to assess the mechanisms behind its effect;

4. To evaluate the protective effect of *E. plantagineum* bee pollen against oxidative stress;

5. To establish possible relationships between the chemical composition and the biological activity of *E. plantagineum* bee pollen.

Chapter II

Experimental Section

1. Standards and reagents

LPS from *Salmonella enterica*, quercetin-3-*O*-rutinoside, lipoxygenase from *Glycine max* (L.) Merr. (type V-S; EC 1.13.11.12), methyl linolelaidate, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), capric, lauric, palmitic, linolenic, stearic, oxalic, aconitic, citric, pyruvic, malonic, shikimic and fumaric acids, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), methanol, Triton X-100, Tris-HCl, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), calcium ionophore A23187, mouse anti-2,4-dinitrophenylated IgE (anti-DNP IgE), albumin from bovine serum (BSA), 2,4-dinitrophenylated BSA (DNP-BSA), 4-nitrophenyl N-acetyl-β-D-glucosaminide, sodium pyruvate, β-nicotinamide adenine dinucleotide reduced form (NADH), 2',7'-dichlorofluorescein diacetate (DCFA-DA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), *tert*-butyl hydroperoxide (*t*-BHP), antipyrene E, phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), perchloric acid, glutathione reduced form (GSH), oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (β-NADPH), 2-vinylpyridine, glutathione reductase (GR, EC 1.6.4.2), 2,4-dinitrophenylhydrazine, 1-chloro-2,4-dinitrobenzene (CDNB), brilliant blue G, diacetylmonoxime (79 mM in 83 mM acetic acid), β-glucuronidase, type H_2 from *Helix pomatia*, sodium linoleate (2 mM in ethanol), potassium hydrogen carbonate, hydrogen peroxide, superoxide dismutase (SOD) bovine (EC 1.15.1.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, delphinidin-3-*O*-glucoside and malvidin-3-*O*-glucoside were obtained from Extrasynthese (Genay, France).

ortho-Phosphoric acid 85% was obtained from Panreac (Barcelona, Spain).

The *n*-alkane series (C_8–C_{40}) was from Supelco (Bellefonte, PA, USA). *N*-(1-Naphthyl)-ethylene-diamine dihydrochloride, sodium nitroprusside (SNP) dehydrate, sodium chloride, methanol, trichloroacetic acid and acetonitrile were obtained from Merck (Darmstadt, Germany) and acetic and sulfuric acids were from Fisher Scientific (Loughborough, UK).

Ethyl acetate, hydrochloric and formic acids were from BDH Prolabo (Dublin, Ireland).

Dulbecco's Phosphate Buffered Saline (DPBS), Dulbecco's Modified Eagle Medium (DMEM), DMEM+GlutaMAX™[1], Earle's Balanced Salt Solution (EBSS), heat inactivated foetal bovine serum (FBS), heat inactivated foetal calf serum (FCS) and Pen Strep solution (Penicillin 5000 units mL^{-1} and Streptomycin 5000 mg mL^{-1}) were obtained from Gibco, Invitrogen™ (Grand Island, NY, USA).

Non-essential aminoacids (NEAA), fungizone (250 μg mL^{-1} amphotericin B), human transferrin (4 mg mL^{-1}) and trypsin-EDTA were purchased from Gibco, Invitrogen™ (Paisley, UK).

Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) media were purchased from Liofilchem (Teramo, Italy).

PGE1, PGE2, tetranor-PGEM, tetranor-PGAM, 20-hydroxy-PGE2, 6-keto-PGF1α, 2,3-dinor-6-keto-PGF1α, 11b-PGF2α, 2,3-dinor-6-keto-PGF1α, 11b-PGF2α, 2,3-dinor-11b-PGF2α, tetranor-PGFM, 9,11-dideoxy-9α,11a-epoxymethano-PGF2α (U-44069), 9,11-dideoxy-9α, 11a-methanoepoxy-PGF2α (U-46619), 11-dehydrothromboxane B2, 8-iso-PGF2α, 2,3-dinor-8-iso-PGF2α, 8-iso-15(R)-PGF2α, 8-iso-15-keto-PGF2α, 8-isoprostaglandin F2α-d4 and 11-dehydro-thromboxane B2-d4 were from Cayman Chemicals (Ann Arbor, Michigan, USA).

Water was treated in a Milli-Q (Miilipore, Bedford, MA, USA) water purification system.

2. Plant material

E. plantagineum bee pollen sample was provided by bee-keepers in the Spanish Extremadura region. The botanical origin was ascertained by Professor Paula B. Andrade (Laboratório de Farmacognosia, Faculdade de Farmácia, Universidade do Porto). Pollen was stored protected from light, under desiccating conditions to prevent alteration. When needed, samples were ground into a fine powder and kept in a desiccator in the dark until analysis.

3. Hydromethanol extract

3.1. Preparation

Bee pollen (0.2 g) was thoroughly mixed with 1 mL methanol-water (7:3), ultrasonicated for 1 h and centrifuged at 2,900 × *g* during 10 min. The supernatant was evaporated under reduced pressure at 40 ºC and the dry residue was stored at −20 ºC, protected from light, until use.

3.2. Alkaloid precipitation tests for alkaloid detection

The presence of alkaloids was discarded by the general alkaloids precipitation tests, adding Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassium mercuric iodide solution) and Bertrand's (silicotungstic acid solution) reagents to the extract.

3.3. HPLC-DAD analysis of phenolic compounds

The extract was analysed in an analytical HPLC unit (Gilson, Villiers-le-bel, France) as before (14), using a LiChroCART column (250 × 4mm, RP-18, 5 μm particle size, LiChrospher®100 stationary phase; Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: water-formic acid (1%) (A) and methanol (B). Elution started with 30% B and a gradient was used to obtain 40% at 20 min, 50% at 25 min, 60% at 30 min and 80% at 32 min. The flow rate was 1 mL min^{-1} and the injection volume 20 μL. Spectral data from all peaks were accumulated in the range 240-600 nm and chromatograms were recorded at 350 nm. The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers-le-Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded at 350 nm relative to external standards. Because standards of all identified flavonoid derivatives were not commercially available, quercetin-3-*O*-neohesperidoside was quantified as quercetin-3-*O*-rutinoside and kaempferol derivatives as kaempferol-3-*O*-rutinoside, except kaempferol-3-*O*-glucoside, which was quantified as itself. Analyses were performed in triplicate.

3.4. HPLC-UV analysis of organic acids

The dry residue of the hydromethanolic extract was dissolved in sulphuric acid 0.01 N. The HPLC-UV analysis was carried out as previously reported (85), in a system consisting of an analytical HPLC unit (Gilson, Villiers-le-bel, France), with an ion exclusion column (Nucleogel Ion 300 OA) in conjunction with a column heating device set at 30 ºC. Elution was carried out isocratically at a solvent flow rate of 0.2 mL min^{-1}, with sulphuric acid 0.01 N. The injection volume was 20 μL. Detection was performed with an UV-Vis detector set at 214 nm. Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The data were processed on a Clarity

Software system (Data Apex, Prague, Czech Republic). Analyses were performed in triplicate.

3.5. GC-IT/MS analysis of fatty acids

3.5.1. Extract purification and derivatization

The extract was purified before fatty acids derivatization. The dry residue of the hydromethanolic extract was thoroughly mixed with acidic water (pH 2 with HCl) and applied to an Amberlite XAD-2 (Fluka Chemicals, Steinheim, Germany: pore size 9 nm, particle size 0.3-1.2 mm) column. Sugars and other polar compounds were eluted with the aqueous solvent. The retained compounds were eluted with ethanol and the internal standard (IS) methyl linolelaidate (100.00 μg mL^{-1} final concentration) was added. The volume was completed to 2.00 mL with ethanol. Fatty acids derivatization was carried out as previously described (86). Briefly, an aliquot of 50 μL of purified extract solution was transferred to a glass vial, the solvent was evaporated under nitrogen stream and 50 μL of derivatization reagent (MSTFA) were added to the dried residue. The vial was capped, vortexed and heated for 20 min in a dry block heater maintained at 60 ºC.

3.5.2. GC-IT/MS conditions

Samples were analysed using a Varian CP-3800 gas chromatographer coupled to a Varian Saturn 4000 mass selective ion trap detector (Palo Alto, CA, USA) and a Saturn GC-MS workstation software (version 6.8). A VF-5 ms (30 m x 0.25 mm x 0.25 μm) column (Varian) and a CombiPAL automatic auto sampler (Varian) were used. The injector port was heated to 250 ºC. Injections were performed in split mode, with a ratio of 1/40. The carrier gas was helium C-60 (Gasin, Leça da Palmeira, Portugal), at a constant flow of 1 mL min^{-1}. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50 and 180 ºC, respectively. The mass ranged from 50 to 600 *m/z*, with a scan rate of 6 scan s^{-1}. The emission current was 50 μA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25.000 μs, with an ionization storage level of 35 *m/z.* The injection volume was 2 μL and the analysis was performed in Full Scan mode. The oven temperature was set at 100 ºC for 1 min, then increasing 20 ºC min^{-1} to 250 ºC and held for 2 min, 10 ºC min^{-1} to 300 ºC and held for 10 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first 4 min to avoid solvent overloading. Compounds were identified by comparison of

retention times and MS fragmentation pattern with those of standards analysed under the same conditions and a mass spectra database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral database. In addition, retention index (RI) was experimentally calculated and the values were compared with those reported in the literature for GC columns with 5%-phenyl-95%-dimethylpolysiloxane (87, 88). For the RI determination, an *n*-alkanes series (C_8-C_{40}) was used. Analyses were performed in triplicate.

3.6. Reactive species scavenging assays in cell-free systems

3.6.1. Superoxide anion ($O_2^{\bullet -}$)

The radical was generated by the PMS/NADH system and monitored by the reduction of NBT to formazan (89). The reaction mixtures consisted of NADH (166 μM), PMS (2.7 μM), NBT (43 μM) and extract (five different concentrations). All components were dissolved in phosphate buffer (19 mM, pH 7.4).

The reaction was conducted at room temperature for 2 min and initiated with the addition of PMS. The absorbance variation was measured spectrophotometrically at 562 nm, in a Multiskan Ascent plate reader (Thermo, Electron Cosporation). Three experiments were performed, in which each concentration was tested in triplicate.

3.6.2. Nitric oxide ($^{\bullet}NO$)

Nitric oxide was generated from SNP and measured by the Griess reagent as before (62). SNP (6 mg mL^{-1}) in phosphate buffer was mixed with different concentrations of bee pollen extract dissolved in the same solvent and incubated at room temperature for 1 h, under light. The nitrite accumulated in the reaction medium was then measured. Equal volumes of reaction medium and Griess reagent (1:1 mixture (v/v) of 1% sulphanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2% H_3PO_4) were mixed and incubated for 10 min in the dark, at room temperature.

The cromophore was determined in a Multiskan Ascent plate reader (Thermo, Electron Cosporation) at 562 nm. Five different extract concentrations were tested. Three experiments were performed, in which each concentration was tested in triplicate.

3.7. Antibacterial capacity

3.7.1. Microorganisms

Nine bacterial species from the American Type Culture Collection (ATCC) were used for the experiments: *Staphylococcus aureus* (ATCC 20231), *Staphylococcus epidermidis* (ATCC 20044), *Salmonella* typhimurium (ATCC 43971), *Proteus mirabilis* (ATCC 4479), *Escherichia coli* (ATCC 30083), *Pseudomonas aeruginosa* (ATCC 50071), *Bacillus cereus* (ATCC 31000), *Enterococcus faecalis* (ATCC 20477) and *Micrococcus luteus* (ATCC 20030). Cultures were obtained from the Laboratory of Microbiology, Faculty of Pharmacy, Porto University. Stock cultures were maintained on MHA at 4 ºC.

3.7.2. Assay

Bacterial inocula were prepared by growing cells in MHB for 24 h, at 37 ºC. Cell suspensions were diluted in sterile MHB to provide final cell counts of 1.56×10^6 colony-forming units *per* mL (CFU mL^{-1}).

Minimum inhibitory concentration (MIC) was determined by employing broth microdilution methods based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, reference documents M07-A8 and M100-S19, with minor modifications (24). Briefly, the suspensions of bacteria cultures were prepared in ampoules containing 2 mL of NaCl 0.85% suspension medium (api®, Biomérieux, Marcy l'Étoile, France). After adjusting the turbidity to 0.5 McFarland, suspensions were diluted in MHB till the final bacterial density of 1.56×10^6 CFU mL^{-1}. The MIC of the extract was determined by two-fold serial dilution method, in 96-well plates: 50 µL of the bacterial suspension was added in each well, which contained 50 µL of the extract dilutions in MHB medium.

The maximum DMSO concentration did not exceed 2.5% (v/v). The plates were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2, without agitation for 18-24 h for both Gram positive and Gram negative bacteria. The MIC was determind as the lowest concentration of the extract inhibiting visual growth of the tested culture on the microplate. Gentamicin MIC for *S. aureus* (ATCC 25923) was determined as quality control. Sterility and positive controls in MHB medium alone and with 2.5% of DMSO (v/v) were included. Positive control wells contained microorganisms without antibiotics. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results.

3.8. Effect on inflammation

3.8.1. Cell culture conditions and treatments

Macrophage RAW 264.7 cells were from ATCC and were kindly provided by Prof. Maria S. J. Nascimento (Laboratory of Microbiology, Faculty of Pharmacy, Porto University). They were cultured at 37 ºC in DMEM supplemented with 10% FBS and 2% Pen Strep solution in a humidified atmosphere of 5% CO_2, as previously reported (49). Cells were seeded in 48-well plates (150 000 cells/ well) and cultured until 80-90% confluence.

Two groups of RAW 264.7 cells were considered: control group (C group) and LPS-treated group (T group). Both C and T groups were cultured in cell media containing growing

doses of bee pollen extract (C0 and T0: 0; C1 and T1: 0.5; C2 and T2: 1.0; C3 and T3: 2.1; C4 and T4: 4.2; C5 and T5: 8.3 mg mL^{-1}; for viability assays concentrations of 16.7 and 33.3 mg mL^{-1} were also tested) dissolved in medium containing 0.5% DMSO. Exposure periods were 19 h for viability (MTT and LDH assays), NO and L-citrulline assays and 9 h for eicosanoids determination. One mg mL^{-1} of LPS was added to the culture media of T group cells after the first hour of extract exposure and this inflammatory stimulus was maintained during the following 8 or 18 h.

3.8.2. MTT reduction assay

This assay evaluates cells' mitochondrial activity, thus allowing determining the number of viable cells. The reaction consists on the reduction of MTT to formazan by mitochondrial dehydrogenase succinate, this enzyme being active only in cells with intact metabolism and respiratory chain (90).

After the incubation period, RAW 264.7 cells were washed with DPBS and then incubated for 30 min with MTT (0.5 mg mL^{-1} in DMEM). The extent of the reduction to formazan within the cells was quantified at 510 nm (69). Results were expressed as percentage of the respective control (with or without LPS). Five independent assays were performed in triplicate.

3.8.3. Lactate dehydrogenase (LDH) leakage

LDH is rapidly released from damaged cells. The consumption of NADH is kinetically measured in the supernatant and is correlated with the amount of extracellular LDH. Thus, cell viability is inversely proportional to the amount of released LDH (91).

Under the assay conditions LDH catalyses the conversion of pyruvate to lactate as NADH is oxidized to NAD^+. The catalytic activity is determined from the rate of disappearance of NADH at 340 nm during 4 min (background correction at 620 nm). Thus, the decrease in absorbance is proportional to LDH activity in the sample (92).

After the treatment period (18 h after LPS addition), an aliquot of the culture medium was taken to determine the activity of LDH leaked through cell membranes, according to a described procedure (69). The cell monolayer was lysed in the remaining medium with lysis buffer (1% triton-X 100 in PBS) to determine total LDH activity. Results are expressed as

LDH activity in media relative to total activity (medium plus cell lysate). Five independent assays were performed in duplicate.

3.8.4. •NO in RAW 264.7 cells culture medium

Nitrite accumulated in the culture medium of C and T-group LPS-stimulated cells was measured after 18 h incubation with LPS. This determination was based on the Griess reaction (69, 93). Equal volumes of culture supernatant and Griess reagent were mixed and incubated for 10 min in the dark, at room temperature. The absorbance was read at 562 nm.

Five independent assays were performed in duplicate.

3.8.5. Determination of L-citrulline

L-citrulline was quantified using the method of Senshu and collaborators (94), with the modifications of Marzinzig and colleagues (95). Briefly, after 18 h incubation with LPS, 100 µL of culture medium of RAW 264.7 cells of C and T-groups were deproteinized with 200 µL of trichloroacetic acid (2.45 M). After centrifugation (10 min; 2,900 x *g*), 250 µL supernatant were mixed with 100 µL of reaction mixture containing 40% (v/v) diacetylmonoxime (79 mM in 83 mM acetic acid), 18% (v/v) antipyrine E (47.8 mM in H_2O) and 42% (v/v) H_2SO_4 7.5 M, and incubated at 96 ºC for 25 min. The solution was then cooled down to room temperature and the absorption was read at 405 nm. Five independent assays were performed in duplicate.

3.8.6. Extraction of eicosanoids from macrophages and culture medium

Eicosanoids were determined in the whole cell extracts and in the culture media of RAW 264.7 cells. The culture medium was collected after 9 h exposure to the extract, 0.005% BHT (final concentration) was added and the medium was kept at −80 ºC until eicosanoids extraction. To precipitate the serum proteins present in the culture media, 3 mL of methanol/HCl 200 mM were added to 2 mL medium and centrifuged at 10,000 x *g* for 5 min.

After removing the culture medium, cells were lysed by incubation with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, containing 0.005% BHT) for 1 h at 0 ºC. Lysates were centrifuged at 8,000 x *g* for 5 min. The resulting supernatants were kept at −80 ºC until eicosanoids extraction.

Before eicosanoids extraction, one replicate of each culture condition was hydrolysed using 5000 UE mL^{-1} of β-glucuronidase (96).

After hydrolysis, both hydrolysed and non-hydrolysed supernatants were subjected to SPE using Strata X-AW cartridge (100 mg $3mL^{-1}$), following the procedure of Medina et al (96). Target compounds were eluted with methanol and dried using a SpeedVac concentrator.

3.8.7. UPLC-QqQ-MS/MS analysis of eicosanoids

The separation of eicosanoids in the cell lysates and culture medium was performed by UPLC coupled to 6460 QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), using the set up of Medina and collaborators (96, 97).

The quantification of the separate eicosanoids detected was performed using the standard compounds PGE1, PGE2, tetranor-PGEM, tetranor-PGAM, 20-hydroxy-PGE2, 6-keto-PGF1α, 2,3-dinor-6-keto-PGF1α, 11b-PGF2α, 2,3-dinor-11β-PGF2α, tetranor-PGFM, 9,11-dideoxy-9α,11α-epoxymethano-PGF2α (U-44069), 9,11-dideoxy-9α,11a-methanoepoxy-PGF2α (U-46619), 11-dehydrothromboxane B2, 8-iso-PGF2α, 2,3-dinor-8-iso-PGF2α, 8-iso-15(R)-PGF2α, 8-iso-15-keto-PGF2α. The isoprostaglandin F2α-d4 (containing 4 deuterium atoms at positions 3, 39,4 and 49) and 11-dehydrothromboxane B2-d4 (containing 4 deuterium atoms at positions 3, 39, 4 and 49) were used as internal standards (96, 97).

3.9. Effect on degranulation

3.9.1. Cell culture conditions and treatments

The rat basophil cell line RBL-2H3 (from ATCC) was grown in DMEM+GlutaMAX™-I supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin, under 5% CO_2 at 37 ºC, in humidified air. For the anti-allergic assays cells were planted at 3×10^5 cells mL^{-1} in 24-wells plates (1 mL/well) and treated after reaching confluence.

In the assay using calcium ionophore as stimulus, cells were pre-treated with the extract (dissolved in culture medium) 15 min prior to the addition of the ionophore (1 µM in EBSS + 0.1% BSA) and maintained in culture for another 30 min. Then the supernatant was collected and used for the determination of β-hexosaminidase release, whereas the MTT cell viability

assay was performed in adherent cells. Three independent assays were performed in duplicate.

In another experiment cells were sensitized with anti-DNP IgE (100 ng mL^{-1}) for 16 h, then stimulated for 1 h with DNP-BSA (100 ng mL^{-1} in EBSS + 0.1% BSA). Cells were co-incubated with the extract throughout the assay. β-Hexosaminidase release and MTT assays were performed as in the assay with calcium ionophore.

The effect of the extract on β-hexosaminidase release in the absence of stimuli (calcium ionophore or IgE/antigen) was simultaneously checked in every assay performed with an allergic stimulus.

3.9.2. MTT reduction assay

Cellular viability was assessed by the reduction of MTT to formazan, according to a previously described method (98), with some modifications. After cell treatment, culture medium removal and cells washing with DPBS, cells in 24-well plates were incubated with 1 mL/well of MTT (0.5 mg mL^{-1}), for 30 min at 37 ºC. Supernatant was then discarded, formazan was solubilized in DMSO (1 mL) and quantified by measurement of optical density at 550 nm, using a microplate reader. Results are expressed as percentage of control (without allergic stimulus). Three independent assays were performed in duplicate with calcium ionophore (or extract only) and six independent assays were performed in duplicate with IgE-DNP – DNP-BSA (or extract only).

3.9.3. Quantification of released β-hexosaminidase

To assess the effect of the extract in RBL-2H3 cells degranulation, β-hexosaminidase was quantified according to a previously published method (99), with some modifications. Briefly, 30 µL of supernatant was incubated with 50 µL of 1.3 mg mL^{-1} 4-nitrophenyl N-acetyl-β-D-glucosaminide (in 0.2 M citric acid, pH 4.5), for 1 h at 37 ºC. β-Hexosaminidase reaction was stopped by the addition of 80 µL of NaOH 0.5 M and absorbance was measured at 405 nm using a microplate reader. Three independent assays were performed in duplicate in the assay with calcium ionophore (or extract only) and six independent assays were performed in duplicate in the assay with IgE-DNP – DNP-BSA (or extract only).

3.9.4. β-Hexosaminidase inhibitory activity

Besides avoiding β-hexosaminidase release, the extract may directly inhibit β-hexosaminidase enzymatic activity. The inhibition of β-hexosaminidase activity was checked by the following assay (99): after culture, cells were lysed by incubation with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) for 1 h, at 0 ºC. Lysates were centrifuged at 8,000 x *g* for 5 min. The resulting supernatants were used to determine β-hexosaminidase activity in the presence and in the absence of the extract, as described in the previous section. Three independent assays were performed in duplicate.

3.9.5. Lipoxygenase inhibition assay

The oxidation of linoleic acid, by lipoxygenase, to the conjugated diene 13-hydroperoxy linoleic acid was followed by measuring the absorbance at 234 nm on an UV/vis spectrophotometer (Helios Alpha, Cambridge, UK). The assay was performed by adding 20 µL of extract dissolved in phosphate buffer (pH 9.0), 1 mL of phosphate buffer and 20 µL of a soybean lipoxygenase solution (500 U). After 5 min incubation, at room temperature, the reaction was started by the addition of 50 µL sodium linoleate (2 mM in ethanol). The reaction time was 3 min and the inhibition of the enzyme activity was calculated by comparing the reaction rate with the control (without extract) (99).

3.10. Statistical analysis

All statistical calculations were made using GraphPad Prism 5 Software (San Diego California, USA). For all cell-system assays, determination of the statistical significance in comparison to control was performed using the parametric method of one-way ANOVA on ranks, followed by Bonferroni's *post*-hoc test in RAW 264.7 macrophages. Two-way ANOVA and Sidak's multiple comparisons test were used to determine the interaction between extract and LPS in cell viability and differences in citrulline and $^{\bullet}NO$ release by LPS-stimulated macrophages.

Two-way ANOVA and Bonferroni's test, as post-hoc test, were used to determine the interaction of the stimulus and the extract in MTT reduction assay in RBL-2H3 cells. Two-tailed *t* paired test was used to determine the statistical significance in comparison to control in β-hexosaminidase release assay.

In all cases, *p* values lower than 0.05 were considered statistically significant.

4. Acidified methanol extract and fractions

4.1. Preparation

Aliquots of 1 g of *E. plantagineum* bee pollen were accurately weighed, thoroughly mixed with 10 mL of methanol containing 0.5% HCl (v/v), ultra-sonicated for 1 h and centrifuged at 4000 rpm during 10 min. The supernatant was decanted and the residue was re-extracted by vortexing for 2 min with 10 mL of methanol containing 0.5% HCl (v/v) and centrifuged at 4000 rpm for 10 min. The supernatants were pooled together and the solvent was evaporated under reduced pressure at 30 ºC. In order to obtain extracts enriched in phenolic compounds, a SPE procedure was performed using Chromabond C_{18} non-end-capped (NEC) columns (45 µm particle size, 60 Å pore size; 10 g sorbent mass, 70 mLreservoir volume) from Macherey-Nagel (Düren, Germany). The residue was dissolved in 10 mL of water and applied to the SPE cartridge preconditioned with 20 mL of ethyl acetate, 20 mL of methanol and 20 mL of water. The loaded cartridge was washed with 10 mL of water. A fraction containing non-coloured phenolics was eluted with 100 mL of ethyl acetate (fraction I). A second fraction with anthocyanins (fraction II) was eluted with 40 mL of methanol. A combined extract was obtained by mixing ethyl acetate and methanol eluates (whole extract). The eluates were concentrated under reduced pressure, and the residues obtained were dissolved in 5 mL of methanol (final concentration of 200 mg mL^{-1}).

4.2. Acid hydrolysis

One mL of HCl 2 M was added to 2 ml of fraction II. The acidic solution was heated at 100 ºC for 30 min, under reflux. The obtained anthocyanins were recovered by SPE using Chromabond C_{18} NEC columns (45 µm particle size, 60 Å pore size; 1 g sorbent mass, 6 mL reservoir volume) from Macherey-Nagel (Düren, Germany), previously conditioned with methanol and HCl 2M. Compounds were eluted with 20 mL of methanol. The solvent was evaporated under reduced pressure at 30 ºC, the residue was dissolved in 2 mL of methanol, filtered through a 0.45 µm pore size filter and injected for anthocyanins identification.

4.3. HPLC-DAD analysis

The extracts were analysed on an analytical HPLC unit (Gilson) equipped with a Spherisorb ODS2 column (25.0 × 0.46 cm, 5 µm, particle diameter). The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

4.3.1. Flavonols

The extracts were analysed according to the method used for the analysis of the hydromethanol extract described in section **3.3.** Spectral data from all peaks were accumulated in the range 200–600 nm, and chromatograms were recorded at 350 nm. Phenolic compounds quantification was achieved by the absorbance recorded at 350 nm relative to external standards. Because standards of all identified flavonoid derivatives were not commercially available, kaempferol derivatives were quantified as kaempferol-3-*O*-rutinoside, except kaempferol-3-*O*-glucoside, which was quantified as itself.

4.3.2. Anthocyanins

Coloured phenolics were analysed following a previously described method (100). The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and water/formic acid/acetonitrile (40:10:50, v/v/v; eluent B), using a gradient program as follows: from 10 to 25% B (10 min), from 25 to 31% B (5 min), from 31 to 40% B (5 min), from 40 to 50% B (10 min), from 50 to 100% B (10 min) and from 100 to 10% B (5 min). The flow rate was 0.8 mL min^{-1} and the injection volume was 20 µL. Chromatograms were recorded at 500 nm. Phenolic compounds in the extracts and in the hydrolysed extracts were identified by comparing their retention times and UV-Vis spectra with those of standards when commercially available and by comparison with literature (37). Quantification was performed at 500 nm. Delphinidin heterosides were quantified as delphinidin-3-*O*-glucoside, petunidin heterosides and malvidin-3-*O*-glucoside were quantified as malvidin-3-*O*-glucoside.

4.4. Effect on cell oxidative stress

4.4.1. Cell culture conditions and treatments

Human colorectal adenocarcinoma cell line (Caco-2) from the American Type Culture Collection (LGC Standards S.L.U., Spain) was grown in DMEM+GlutaMAX^{TM}-I supplemented with 10% foetal bovine serum (FBS) heat-inactivated, 1% non-essential aminoacids, 1% fungizone and 6 µg mL^{-1} transferrin, 1% penicillin and 1% streptomycin, at 37 ºC in 5% CO_2, until they reach 90% confluence. For cells treatment the culture medium was prepared without FCS. Cells were plated at 1.5×10^5 cells mL^{-1} using 200 µL/well of cell suspension in 96-well plates (viability assays), 1200 µL/well in 24-well plates (GSH, DCFA-DA) and 2.5 mL/well in 12-well plates (enzymes) and then incubated until they reached confluence.

Just before the assays, an aliquot of the extracts was evaporated under a nitrogen stream, the residue was dissolved in the same volume of culture medium without FCS and sterilized by filtration through a 0.22 µm size pore membrane. A series of five dilutions was prepared and cells were exposed for 24 h to the different concentrations of the extracts. In order to evaluate the antioxidant potential, cells were pre-exposed for 24 h to the extracts, then the extracts were removed and cells were exposed to 150 µM of *t*-BHP for 4 (DCFA-DA assay) or 6 h (other assays).

4.4.2. MTT reduction assay

After cells treatment and removal of the culture medium, adherent cells were treated for 30 min with 0.5 mg mL^{-1} of MTT. The formazan crystals were then dissolved with DMSO and quantified by measuring the absorbance at 560 nm in a microplate reader (98). Results are expressed as percentage of control. Five independent experiments were performed in triplicate.

4.4.3. LDH leakage

LDH was determined by adding pyruvate (124 µM) and NADH (154 µM) to the culture medium of treated cells and following the rate of oxidation of NADH at 340 nm during the conversion of pyruvate to lactate (98, 101). Results are expressed as percentage of control. Five independent experiments were performed in triplicate.

4.4.4. Reactive species

The measurement of intracellular reactive species with DCFA-DA was performed using a previously described method with modifications (102). Cells were exposed to the extracts for 24 h and the culture medium was removed. DCFA-DA (10 µM, dissolved in culture medium (DMEM) without phenol red) was added and the cells were incubated with the probe for 30 min at 37 ºC. The probe was removed and *t*-BHP dissolved in culture medium without phenol red was added. Fluorescence (485 nm excitation, 528 nm emission) was read immediately and every 30 min during 4 h, in a microplate spectrofluorimeter (Synergy HT, Bio-Tek Instruments Winooski, USA, operated by KC4 software). The plate was maintained at 37 ºC between readings. Results are expressed as % of control (without *t*-BHP) and were normalized with the result at 0 time. Five independent assays were performed.

4.4.5. Total glutathione

Total glutathione (GSHt) was measured by DTNB-glutathione reductase recycling assay, according to a previously described method (103, 104). In brief, after cells treatment, the culture medium was removed and 250 µL of perchloric acid 5% were added to each well. Incubation was maintained during 10 min on ice in order to extract GSHt, then the solution was collected and kept at −20 ºC until analysis. 250 µL of NaOH 0.3 M were added to the cells in each well to solubilize proteins for protein quantification.

GSH was measured by following the increase of 5-thio-2-nitrobenzoic acid (TNB) at 405 nm, resulting from the oxidation of GSH by DTNB (1.28 mM). The assay was performed after neutralizing perchloric acid with $KHCO_3$ 0.76 M, in the presence of GR (2.0 U mL^{-1}) and NADPH (0.21 mM), to recycle GSSG, increasing the detection limit. Five independent assays were performed.

4.4.6. Antioxidant enzymes

For the measurement of enzymes activity, after exposure to the extracts the culture medium was collected for extracellular SOD analysis and the cells were lysed on ice for 30 min with 1% Triton X-100 in Tris 50 mM pH=8.0, containing 150 mM NaCl, 0.5 mM PMSF and 1 mM DTT (for GPx analysis 0.5 mM of DTT was used). The lysates were centrifuged at 10,000 rpm for 10 min at 4 ºC and the supernatants were collected for enzymatic and protein assays. Five independent assays were performed.

4.4.6.1. Glutathione-S- transferase

Glutathione-S-transferase (GST) activity was assayed according to a previously described method (105). GSH (1 mM) was incubated at room temperature with CDNB (1.5 mM) and the formation of the conjugate was monitored at 340 nm. GST activity was calculated by using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

4.4.6.2. Glutathione reductase

Glutathione reductase (GR) activity was determined at room temperature, following the decrease of NADPH (0.5 mM) at 340 nm, during the reduction of GSSG (1.2 mM) to GSH (106). GR activity was calculated by using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

4.4.6.3. Glutathione peroxidase

Glutathione peroxidase (GPx) activity was determined following a previously described method (107). The activity was indirectly measured by a coupled assay of GSSG reduction, under conditions in which GPx activity is rate limiting. The assay was performed at room temperature using *t*-BHP (0.15 mM) as substrate, GSH (1 mM) plus NADPH (0.15 mM) and GR (0.03 U mL^{-1}) to recycle the formed GSSG. The decrease of NADPH was measured at 340 nm. The enzyme activity was calculated by using an extinction coefficient of 6.22 mM^{-1} cm^{-1} and expressed as mmol of NADPH oxidized/min/mg of protein.

4.4.6.4. Catalase

Catalase activity was measured at room temperature by monitoring the decomposition of H_2O_2 (10 mM) at 240 nm (107). The enzyme activity was calculated by using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

4.4.6.5. Superoxide dismutase

Extracellular SOD was assayed using a previously described method (107) with modifications. A PMS (0.003 mM) / NADH (0.07mM) system was used to generate $O_2^{\bullet-}$ and subsequent reduction of NBT (0.03 mM) by $O_2^{\bullet-}$ was monitored at 560 nm. SOD activity was

determined by interpolation in a calibration curve, built under the same conditions with bovine SOD in the concentration range of 0.025- 0.25 U mL^{-1}.

In order to screen for $O_2^{\bullet-}$ scavenging activity, an assay was performed by incubating culture medium without cells, and measuring NBT reduction in the previously described conditions. Four independent assays were performed. The results are expressed as % of control and compared with the ones obtained in the assay performed with cells.

4.4.7. Protein quantification

Protein quantification was performed according to the Bradford method (108). Proteins were measured by addition of 200 mL of Bradford dye reagent (brilliant blue G, 0.1 mg mL^{-1}; ethanol, 5% (v/v); phosphoric acid, 10 % (v/v); water) to 40 mL of the acidified extract or fractions I and II, pre-diluted 100 times. The photometrical measure was performed at 595 nm. Bovine serum albumin was used to generate a standard curve (34).

4.5. Statistical analysis

All statistical calculations were made using Graphpad Prism 6 Software (San Diego, CA, USA). For all cell systems assays, the statistical significance in comparison to control was estimated using the parametric method of two-way ANOVA, followed by Dunnets's test (LDH, MTT, DCFA-DA and antioxidant enzymes) or Sidak's (GSHt, superoxide), as post-hoc test. Two-way ANOVA and Sidak's multiple comparisons test were used to compare between groups.

In all cases, *p* values lower than 0.05 were considered statistically significant.

Chapter III

Results and Discussion

1. Hydromethanol extract

1.1. Chemical composition

As referred above, pollen from *Echium* species are described to be a potential source of pyrrolizidine alkaloids, a class of secondary metabolites with hepatotoxic properties. The presence of alkaloids in *E. plantagineum* bee pollen hydromethanolic extract was screened by the general alkaloids precipitation tests (Dragendorff's, Mayers's and Bertrand's), giving negative results.

1.1.1. Phenolic profile

The phenolic profile of *E. plantagineum* bee pollen hydromethanolic extract was established by HPLC-DAD (**Figure 15**). The chromatogram showed kaempferol-3-*O*-neohesperidoside (**4**) as a major peak, followed by its acylated derivative, kaempferol-3-*O*-(3'/4'-acetyl)-neohesperidoside (**6**), and eight minor peaks, comprising both quercetin and kaempferol derivatives.

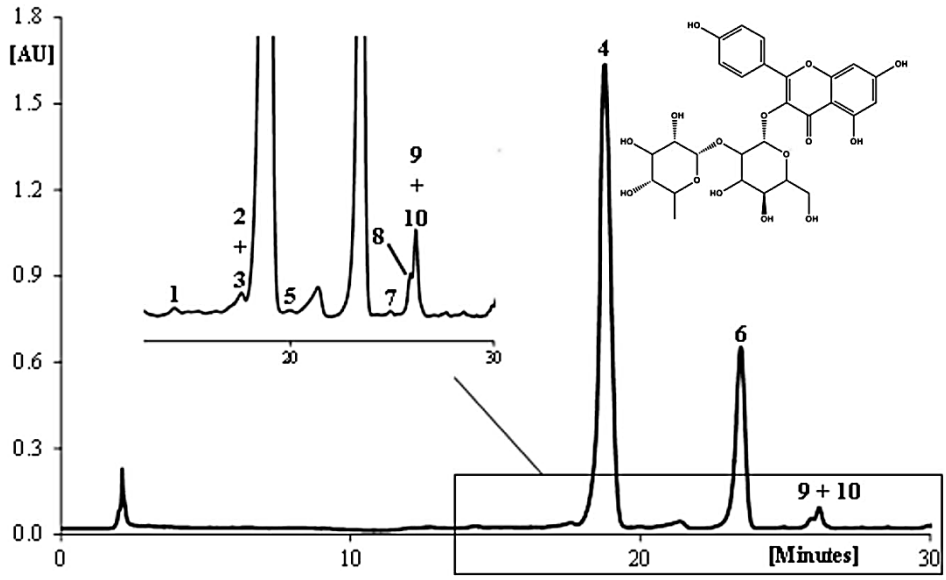


Figure 15. HPLC-DAD chromatogram (350 nm) of phenolic compounds in *E. plantagineum* bee pollen hydromethanolic extract and chemical structure of the main compound, kaempferol-3-*O*-neohesperidoside (**4**). Other compounds: (**1**) quercetin-3-*O*-neohesperidoside; (**2**) kaempferol-3-*O*-sophoroside; (**3**) kaempferol-3-*O*-(3'/4'-rhamnosyl) neohesperidoside; (**5**) kaempferol-3-*O*-neohesperidoside derivative; (**6**) kaempferol-3-*O*-(3'/4'-acetyl)neohesperidoside; (**7**) kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside; (**8**) kaempferol-3-*O*-glucoside; (**9**) kaempferol-3-*O*-rutinoside and (**10**) kaempferol-3-*O*-(3'/4'-acetyl)-neohesperidoside isomer.

The content of compounds **4** and **6** was ca. 9 and 3 g kg^{-1}, respectively. The other phenolics accounted together for 0.4 g kg^{-1} (**Table 3**).

Table 3. Phenolic compounds content in *E. plantagineum* bee pollen hydromethanol extract.

	Phenolic compounds	**mg kg^{-1} (Dry pollen)[a]**
1	Quercetin-3-*O*-neohesperidoside	24.8 ± 0.1
2+3	Kaempferol-3-*O*-sophoroside + kaempferol-3-*O*-(4''-rhamnosyl)-neohesperidoside	120.9 ± 0.1
4	Kaempferol-3-*O*-neohesperidoside	8864.1 ± 14.3
5	Kaempferol-3-*O*-neohesperidoside derivative	25.8 ± 0.6
6	Kaempferol-3-*O*-(3'/4'-acetyl)-neohesperidoside	2988.1 ± 10.0
7	Kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside	20.7 ± 0.7
8	Kaempferol-3-*O*-glucoside	15.1 ± 0.1
9+10	Kaempferol-3-*O*-rutinoside + kaempferol-3-*O*-(3'/4'-acetyl)-neohesperidoside isomer	213.6 ± 2.1
	Total	**12273.2**

[a] Values are expressed as mean ± standard deviation of three determinations.

1.1.2. Organic acids profile

Eight organic acids were identified in *E. plantagineum* bee pollen hydromethanol extract: oxalic, aconitic, citric, pyruvic, malonic, shikimic, acetic and fumaric acids (**Figure 16**).

The sum of the determined organic acids was ca. 10 g kg^{-1} (**Table 4**), malonic acid (**5**) being the main compound (ca. 80% of total identified organic acids). Fumaric acid (**8**) was the minor compound (ca. 0.005% of the total acids).

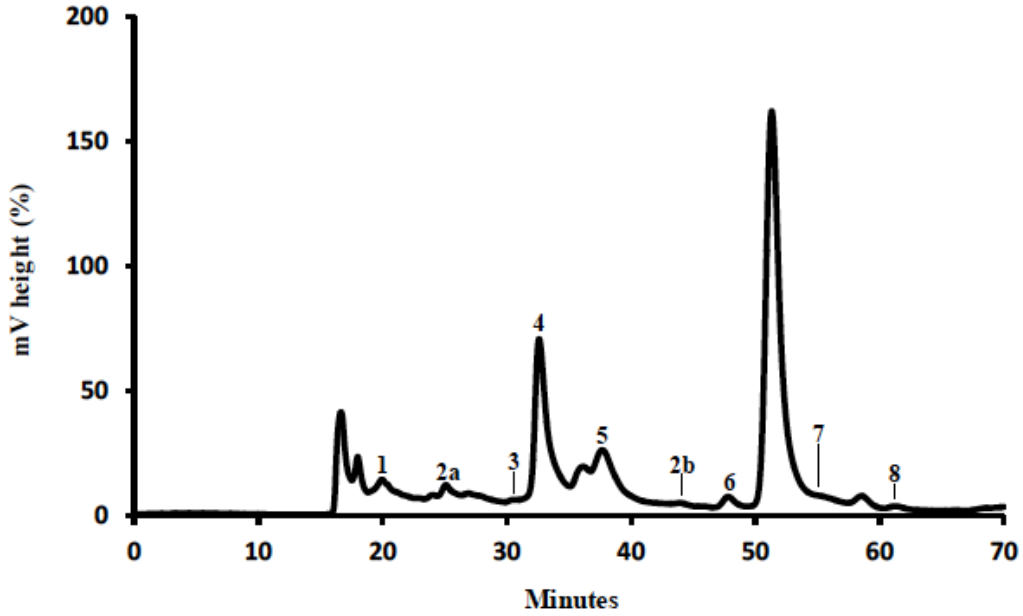


Figure 16. HPLC-UV organic acids profile of *E. plantagineum* hydromethanol extract. Detection at 214 nm. Compounds: oxalic acid (**1**), aconitic acid (**2a+2b**), citric acid (**3**), pyruvic acid (**4**), malonic acid (**5**), shikimic acid (**6**), acetic acid (**7**) and fumaric acid (**8**).

Table 4. Organic acids content of *E. plantagineum* bee pollen hydromethanol extract.

Organic acid		mg kg^{-1} (Dry pollen)[a]
1	Oxalic	51.05 ± 0.98
2a + 2b	Aconitic	6.89 ± 0.26
3	Citric	10.24 ± 0.05
4	Pyruvic	890.27 ± 0.85
5	Malonic	8152.87 ± 0.37
6	Shikimic	2.20 ± 0.43
7	Acetic	1132.02 ± 111.33
8	Fumaric	0.48 ± 0.02
	Total	10245.54 ± 114.29

[a] Results are expressed as mean ± standard deviation of three determinations.

1.1.3. Fatty acids profile

Five fatty acids were determined in *E. plantagineum* bee pollen hydromethanol extract after purification, performed in order to protect the equipment and facilitate identification (**Figure 17**).

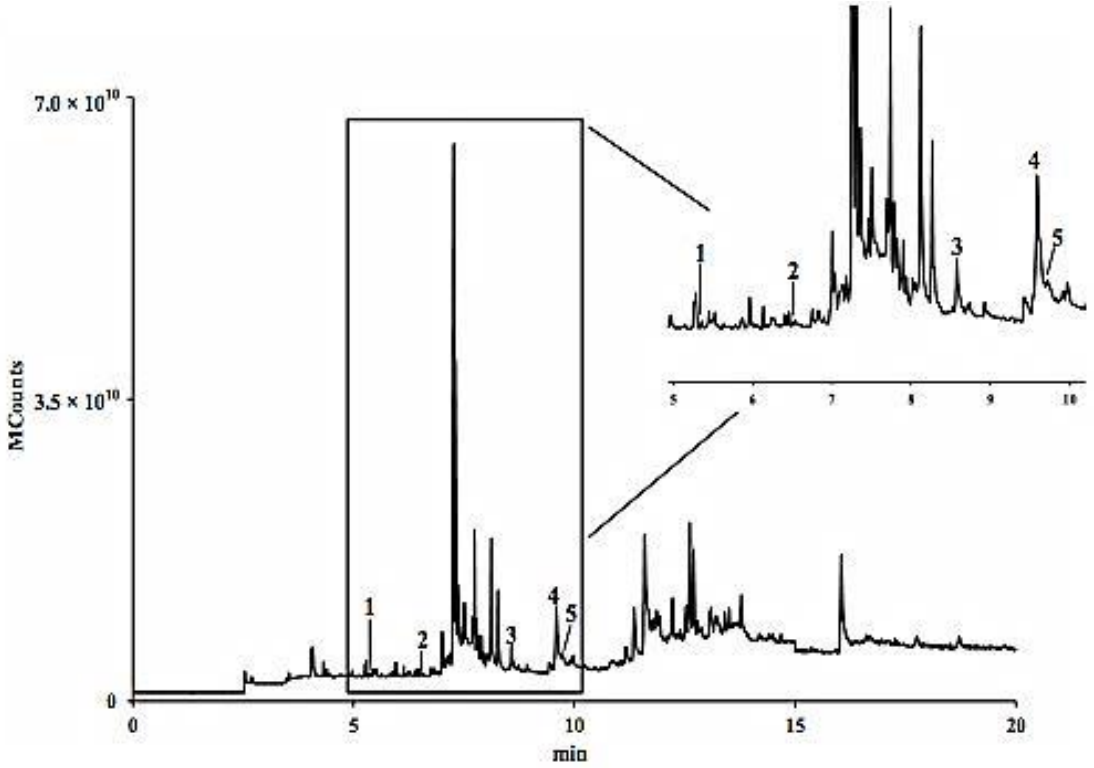


Figure 17. GC-IT/MS fatty acids profile of *E. plantagineum* hydromethanol extract. All compounds correspond to their trimethylsilyl (TMS) derivatives: capric acid (**1**), lauric acid (**2**), palmitic acid (**3**), α-linoleic acid (**4**) and stearic acid (**5**).

The fatty acids content was *ca.* 690 mg kg^{-1}, palmitic acid being the major compound (ca. 57%), followed by α-linolenic acid, which represented ca. 27% of the identified compounds (**Table 5**).

Table 5. Fatty acid content of *E. plantagineum* bee pollen hydromethanol extract.

Fatty Acid		mg kg^{-1}(Dry Pollen)[a]
1	Capric (C10:0)	53.0 ± 1.7
2	Lauric (C12:0)	27.1 ± 2.6
3	Palmitic (C16:0)	391.6 ± 19.3
4	α-Linolenic (C18:3)	183.9 ± 18.6
5	Stearic (C18:0)	34.8 ± 2.8
	Total	**690.4 ± 45.0**

[a] Results are expressed as mean ± standard deviation of three determinations

Although the fatty acids profile of bee pollen hydromethanol extract is different from the ones previously described in *E. plantagineum* seeds (109) and leaves (61) oils, α-linolenic acid is a representative fatty acid in all plant materials.

1.2. Biological effects

Inflammation is a protective immunovascular response against harmful stimuli. As it is a defence mechanism, it manifests in most of the diseases, like allergic and infectious diseases. In this sense, the hydromethanol extract of *E. plantagineum* was studied as to determine its antioxidant potential, anti-inflammatory, anti-bacterial and anti-allergic effect.

1.2.1. Reactive species scavenging in cell-free systems

Before testing in macrophages, the hydromethanol extract of *E. plantagineum* bee pollen was screened for antioxidant activity against $^{\bullet}NO$ and $O_2^{\bullet-}$ in cell-free systems. The extract scavenged the sodium nitroprusside-generated $^{\bullet}NO$ in a concentration dependent manner (IC_{25} = 1.9 mg mL^{-1}) (**Figure 18**).

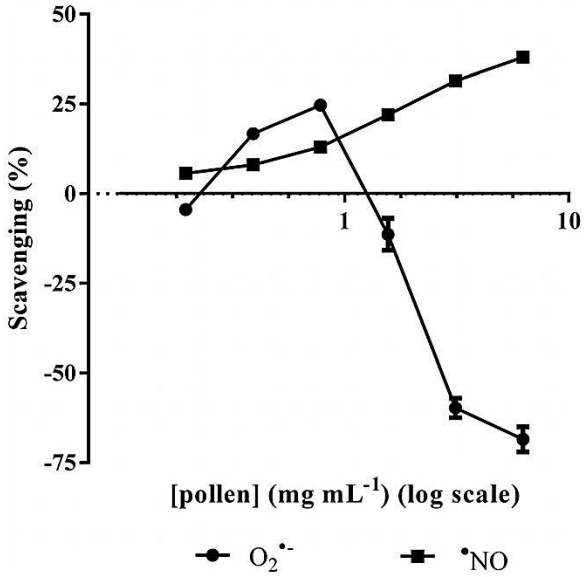


Figure 18. Effect of *E. plantagineum* bee pollen hydromethanol extract against $^{\bullet}NO$ and $O_2^{\bullet-}$ in cell-free systems. Results expressed as mean ± SEM of three independent experiments performed in triplicate.

However, a dual behaviour was seen against $O_2^{\bullet-}$ generated by the PMS/NADH system: until 0.8 mg mL^{-1} a dose-dependent scavenging effect was observed, but a pro-oxidant response was noticed with higher concentrations, the compounds present seeming to participate in the formation of $O_2^{\bullet-}$ (**Figure 18**).

The direct interaction of the compounds present in the extract with NBT was excluded, because the reduction of NBT to formazan did not occur when the assay was performed in the absence of PMS and NADH. Furthermore, we can also assume that the superoxide is not generated by the direct oxidation of kaempferol derivatives by molecular oxygen. So, the apparent pro-oxidant behaviour at higher extract concentrations observed in this system for evaluating $O_2^{\bullet-}$ scavenging potential can be without biological significance, since PMS and NBT are not endogenous molecules. Nevertheless a pro-oxidant behaviour has been previously observed with flavonoids and flavonoid-rich extracts (110, 111).

1.2.2. Effect on inflammation

The cellular model of LPS-stimulated macrophages is widely used to assess anti-inflammatory activity (68, 69, 112). It is well known that infection of cells by microorganisms activates the inflammatory response due to the recognition by macrophages receptors of LPS present in microorganisms' cell wall: the initial sensing of infection is mediated by innate pattern recognition receptors that are expressed by

macrophages dendritic cells and various nonprofessional immune cells. Although inflammation is a protective response of the body to ensure removal of detrimental stimuli, as well as a healing process for repairing damaged tissue, the stimulation of macrophages, initiated by microorganisms (or other agent) can result in the overproduction of inflammatory mediators like PGE2 and PGI2, which enhance edema formation and leukocyte infiltration by promoting blood flow in the inflamed region. This process can be at the origin of inflammatory diseases like type 2 diabetes, atherosclerosis and Alzheimer's disease (72).

1.2.2.1. Effect on cell viability

The extract was not cytotoxic until 8.3 mg mL^{-1} (equivalent to 172 mM of kaempferol and quercetin derivatives). The exposure to 16.7 mg mL^{-1} for 19 h induced a significant decrease in cell viability (p<0.01), as assessed by the MTT assay. The cellular viability was further decreased by the extract at 33.3 mg mL^{-1}, being statistically significant in both LDH and MTT assays (p<0.001) (**Figure 20**).

Cell treatment with 1 mg mL^{-1} LPS did not significantly alter cell viability and the results obtained with cells exposed to extract and LPS were similar to the ones found with cells exposed only to the extract (p<0.05) (**Figure 19**). In fact, for the concentration of 8.33 mg mL^{-1} an increase in viability was shown (p<0.01).

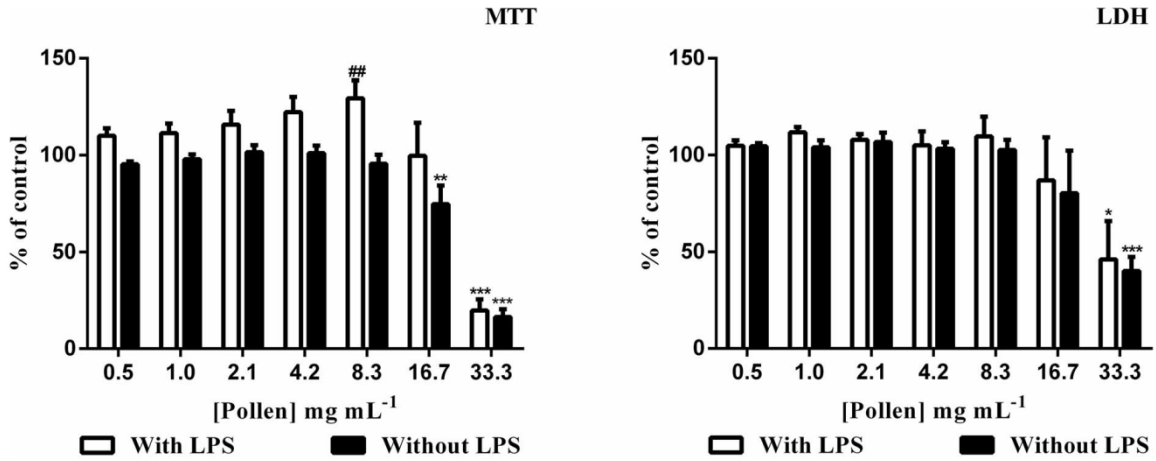


Figure 19. Effect of *E. plantagineum* bee pollen extract in cell viability. RAW 264.7 macrophages were pre-exposed to the extract for 1 h followed by 18 h co-exposition with 1 mg mL^{-1} of LPS. Cell viability was assessed by MTT reduction and LDH release assays. Values show mean ± SEM of five independent assays performed in duplicate. *p<0.05; **p<0.01; ***p<0.001, compared to the respective control; ##p<0.01 LPS- exposed cells compared with cells exposed only to the respective extract concentration.

1.2.2.2. •NO and L-citrulline in culture medium

The levels of •NO and L-citrulline in the culture medium of LPS stimulated cells (T group) after 18 h exposure were 11.8±1.7 and 10.8±0.9 μM, respectively. The levels of these metabolites in the C group were below the quantification limit. *E. plantagineum* bee pollen hydromethanol extract at non-toxic concentrations dose-dependently decreased •NO in the culture medium of LPS-stimulated cells (p<0.001) (**Figure 20**). Accordingly, the formation of L-citrulline was also inhibited by the extract at all concentrations, being significant for concentrations higher than 1.0 mg mL^{-1} (p<0.001) (**Figure 20**).

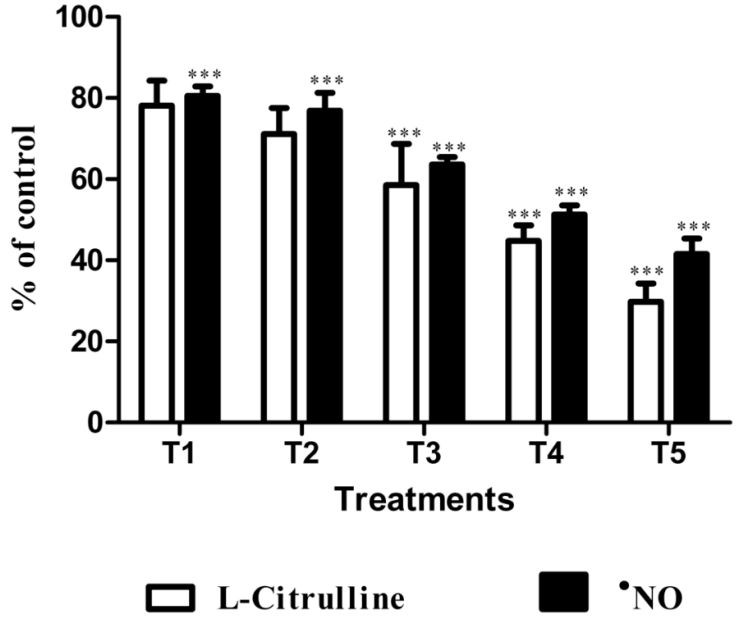


Figure 20. Effect of *E. plantagineum* bee pollen extract pre-exposure in •NO and L-citrulline production by LPS-stimulated macrophages. RAW 264.7 macrophages were pre-exposed to the extract for 1 h followed by 18 h co-exposition with 1 mg mL^{-1} of LPS (T-group). Extract concentrations: T0: 0; T1: 0.5 mg mL^{-1}; T2: 1.0 mg mL^{-1}; T3: 2.1 mg mL^{-1}; T4: 4.2 mg mL^{-1}; T5: 8.3 mg mL^{-1}. Values show mean 6 SEM of five independent assays performed in duplicate. ***p<0.001, compared to LPS-only exposed cells.

In relation to •NO, LPS induces its production by iNOS accompanied by L-citrulline formation in stoichiometric amounts (113). Accordingly, the decrease of the radical was paralleled by the decrease of L-citrulline in the culture medium of LPS-stimulated macrophages exposed to the extract (**Figure 20**). Given these results, we can state that the extract inhibits iNOS induction by LPS.

1.2.2.3. Effect on eicosanoids

Besides •NO, LPS-stimulated macrophages produce PGs as a consequence of COX-2 up-regulation. Attending to the evanescence of primary PGs, we proceeded to the measurement of some metabolites by UPLC-MS. As isoprostanes (IsoPs) suffer glucuronidation (97, 114) and UDP-glucuronosyltransferases are present in macrophages (115), an hydrolysis step was included in the eicosanoids extraction protocol to ensure that all eicosanoids found in the macrophages lysate and/or cell culture supernatants were totally determined by UPLC-MS and to avoid underestimations of their concentrations (96, 97).

The levels of prostaglandins and IsoPs were measured in RAW macrophages whole-cell lysate, under mild conditions (**Figure 21**). Two prostaglandins (PGE2 and 11β-PGF2α) were quantified both in control cells and in cells exposed to LPS, but higher levels were observed in the latter (**Figure 21A**). The levels of PGE2 (about 1 ng mL^{-1}) in non-stimulated cells were not significantly affected by bee pollen extract. The increased levels of PGE2 in LPS-stimulated cells were reduced by the extract at 0.5 and 1 mg mL^{-1} (p<0.01), but for higher concentrations the results were not significantly different from those of cells only exposed to LPS (**Figure 21A**).

In what concerns to 11β-PGF2α, the extract showed a tendency to increase the levels of this PGD2 metabolite in control cells. In LPS exposed cells the levels of 11β-PGF2α were dose-dependently decreased by the extract at 0.5 and 1 mg mL^{-1}, but raised for higher concentrations, being similar to that found in LPS-only exposed cells for the highest concentration tested (**Figure 21A**).

Besides prostaglandins, three IsoPs (8-iso-15(*R*)-PGF2α, 8-iso-15-keto-PGF2α and 8-iso-PGF2α) were quantified in the cellular lysate (**Figure 21B**). Their contents in control cells were increased by the extract. LPS itself raised the levels of isoprostanes inside the cell and, in general, bee pollen extract decreased IsoPs below the basal level at 1 mg mL^{-1}. Higher extract concentrations raised IsoPs levels (**Figure 21B).**

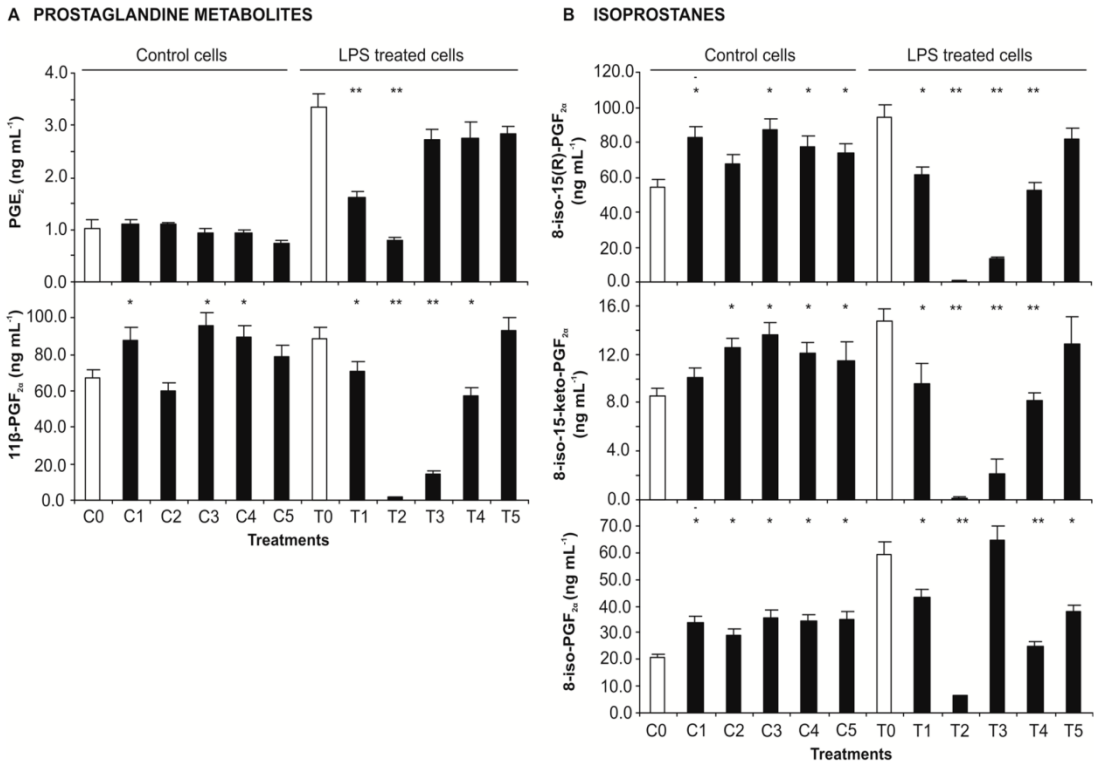


Figure 21. Effect of *E. plantagineum* bee pollen extract pre-exposure in intracellular eicosanoids levels of LPS-stimulated macrophages. Eicosanoids were determined in the whole cell extracts of RAW 264.7 macrophages pre-exposed to the extract for 1 h, followed by 8 h co-exposition with 1 mg mL^{-1} of LPS. C group: control group exposed to extract only. T group: extract and LPS co-exposed group. Extract concentrations: C0 and T0: 0; C1 and T1: 0.5 mg mL^{-1}; C2 and T2: 1.0 mg mL^{-1}; C3 and T3: 2.1 mg mL^{-1}; C4 and T4: 4.2 mg mL^{-1}; C5 and T5: 8.3 mg mL^{-1}. Values show mean ± SEM of two independent experiments performed in triplicate. (**A**) Prostaglandin and prostaglandin metabolites. (**B**) Isoprostanes. *p<0.05; **p<0.01 compared to the respective control (with or without LPS).

The action of COX-2 on AA leads to the formation of PGH2, the substrate for the various PG synthases (**Figure 22**). *In vivo* studies have shown that COX-2 inhibitors reduce PGE2 more profoundly than other PGs (116). PGE2 was the prostaglandin with the highest increase in the whole cell lysate of LPS-stimulated macrophages (**Figure 21**). The selective increase of this PG in LPS-stimulated rat macrophages is probably due to the up-regulation of PGE2 synthase in concert with COX-2 (117, 118).

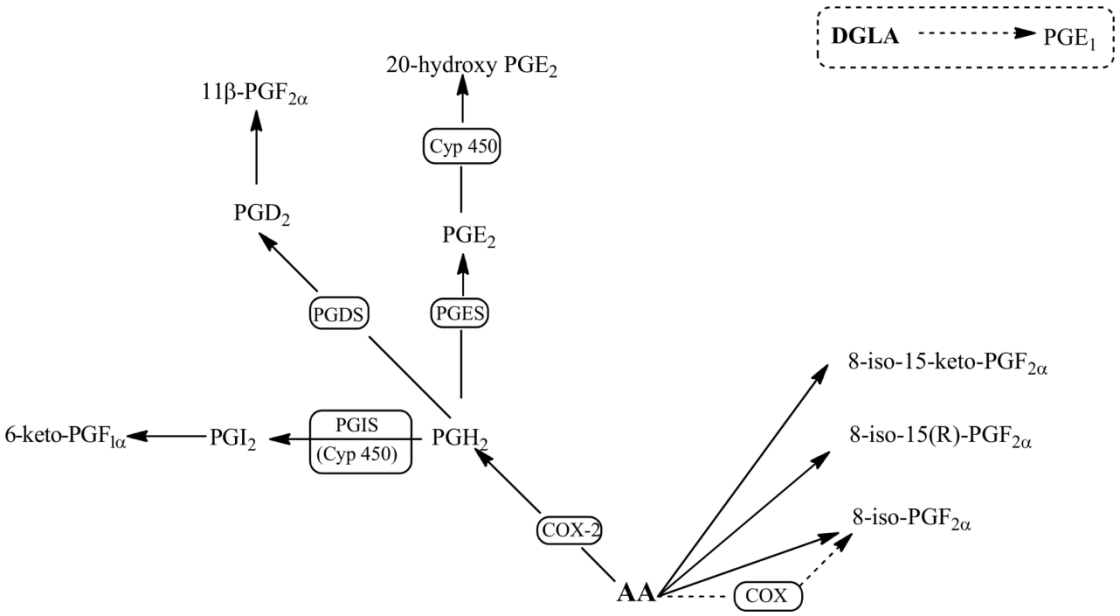


Figure 22. Metabolic pathways of eicosanoids determined in LPS-stimulated macrophages pre-exposed to *E. plantagineum* bee pollen extract. AA, arachidonic acid; COX, cyclooxygenase; Cyp 450, Cyt P450 enzyme; DGLA, dihomo-c- linolenic acid; PG, prostaglandin; PGS, PG synthase.

Additionally, the eicosanoids profile in the culture medium of RAW 264.7 macrophages was also established (**Figure 23**). Two prostaglandins (PGE1 and PGE2) and three prostaglandins metabolites (6-keto-PGF1α, 20-hydroxy PGE2 and 11β-PGF2α) were determined (**Figure 23A**). PGE1 release to the medium was increased by LPS exposure and the extract at all concentrations tested was able to decrease the levels of this PG. PGE1 returned to the levels observed in the culture medium of non-stimulated cells, for extract concentrations higher than 0.5 mg mL^{-1} (**Figure 23A**).

Also PGE2 release was highly increased by LPS exposure (**Figure 23A**). Although this prostaglandin was also measured in non-stimulated cells, a 20 fold increase was observed after 8 hours exposure to LPS. The extract decreased PGE2 release into the medium at 1 mg mL^{-1} in control cells and at 0.5 mg mL^{-1} in LPS exposed cells. For higher extract concentrations the levels of PGE2 in the T group were higher than the ones found in LPS-only exposed cells ($p<0.05$). On the other hand, considerable amounts of 20-hydroxy PGE2 were found only in the medium of LPS exposed cells and LPS plus extract at 0.5 mg mL^{-1}

and 1 mg mL^{-1}. A similar behaviour was observed for 6-keto-PGF1α, a PGI2 metabolite that was only quantified in the medium of LPS exposed cells and LPS plus extract at 0.5 mg mL^{-1}.

Considering 11β-PGF2α, the amount of this PG metabolite was higher in the culture medium of non-stimulated cells, compared to LPS-stimulated cells. Particularly in LPS-stimulated RAW macrophages, the extract did not have a significant effect on the release of 11β-PGF2α, except when at 2.1 mg mL^{-1}.

In what concerns to IsoPs, it was observed that both 8-iso-15(*R*)-PGF2α and 8-iso-15-keto-PGF2α increased with LPS exposure, while the levels of 8-iso-PGF2α decreased (**Figure 23B**). The effect in the former IsoPs was significantly reduced by the extract only at the lowest concentration tested ($p<0.05$). However, in control cells, the extract at 0.5 mg mL^{-1} significantly increased the levels of 8-iso-15(*R*)-PGF2α, 8-iso-15-keto-PGF2α, as well as that of the prostaglandin metabolite 11β-PGF2α.

Regarding 8-iso-PGF2α, the extract at 1 mg mL^{-1} further decreased its level in LPS-exposed cells.

Although PGF2α has been implicated in acute inflammation, in this model of inflammation, tetranor-PGFM, which is PGF2α main metabolite, was not detected, neither in whole cell extract nor in the culture medium.

U-44069, an analogue of PGH2, thromboxane A2 (as its stable analogue U-46619) and 11-dehydrothromboxane B2 were not encountered in whole cell lysates or culture medium of macrophages.

The search for 2,3-dinor-6-keto-PGF1α (a PGI2 metabolite) and the IsoP 2,3-dinor-8-iso-PGF2α in the cell lysate and culture medium gave negative results.

Comparing the levels of eicosanoids in the culture medium and inside the cell (**Figures 21** and **23A** and **23B**) it can be observed that eicosanoids are mainly secreted into the medium, with the exception of 11β-PGF2α and 8-iso-PGF2α that are present at similar levels inside and outside the cell.

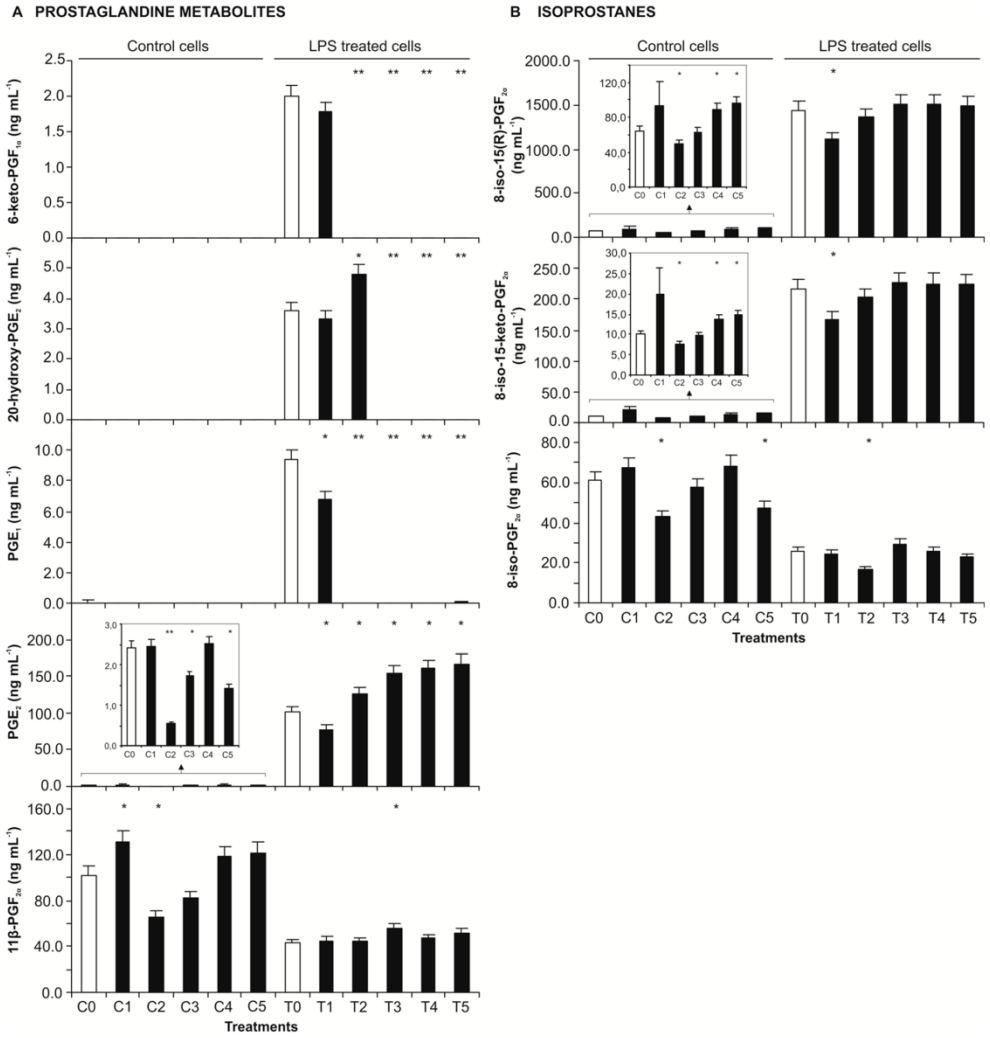


Figure 23. Effect of *E. plantagineum* bee pollen extract pre-exposure in eicosanoids release of LPS-stimulated macrophages. Eicosanoids were determined in the culture media of RAW 264.7 macrophages pre-exposed to the extract for 1 h followed by 8 h co-exposition with 1 mg mL^{-1} of LPS. C group: control group exposed to extract only. T group: extract and LPS co-exposed group. Extract concentrations: C0 and T0: 0; C1 and T1: 0.5 mg mL^{-1}; C2 and T2: 1.0 mg mL^{-1}; C3 and T3: 2.1 mg mL^{-1}; C4 and T4: 4.2 mg mL^{-1}; C5 and T5: 8.3 mg mL^{-1}. Values show mean ± SEM of two independent assays experiments performed in triplicate. (**A**) Prostaglandin and prostaglandin metabolites. (**B**) Isoprostanes. **p*<0.05; ***p*<0.01 compared to the respective control (with or without LPS).

PGE2 undergoes hydroxylation at C-20 catalysed by Cyt P450 enzymes, originating 20-hydroxy PGE2 (119). This PGE2 metabolite was released to the culture medium of macrophages in response to LPS stimulation, but the extract at concentrations higher than 1 mg mL^{-1} decreased the release of 20-hydroxy PGE2 below the quantification limits, as observed in the culture medium of non-exposed cells (**Figure 23**)**.** Therefore, the extract at 1 mg mL^{-1} was able to neutralize the toxic effect exerted by LPS. This result can be attributed to the known inhibition of Cyt P450 enzymes by flavonoids (120). In opposition, the effect of the extract on PGE2 was not so pronounced, being more effective at lower

concentrations (**Figures 21** and **23**). Tetranor-PGEM and tetranor-PGAM, PGE2 metabolites (121), were not detected.

PGE1, a PG derived from dihomo-c-linolenic acid (**Figure 22**), was found in the culture medium of LPS stimulated macrophages. The extract was more effective in decreasing the release of PGE1 than of PGE2 (**Figure 23**).

As said above, another prostanoid found in the culture medium of cells exposed to LPS was 6-keto PGF1α, a PGI2 metabolite (**Figure 23**). PGI2 formation is mediated by prostacyclin synthase, a member of the Cyt P450 monooxygenase family. PGI2 is readily hydrolysed to form the stable 6-keto PGF1α (121). Interestingly, cells treated with the lowest extract concentration did not significantly alter the concentration of this metabolite, although for all the other extract concentrations 6-keto PGF1α levels were below quantification limits. So, it is possible that the phenolic compounds present in the extract exert an inhibitory effect on prostacyclin synthase, under inflammatory conditions, as seen for the inducible form of NOS.

Thromboxane A2 biosynthesis is also catalysed by a member of the Cyt P450 enzymes and it counteracts PGI2 action (121). The absence of thromboxane A2 and its metabolite could be expected, since thromboxane A2 formation is mediated by COX- 1, although in LPS-stimulated cells COX-2 can also be involved (117).

PGD2 has important roles in the mediation and resolution of inflammation. This PG is converted to several metabolites, including 11β-PGF2α, which maintains the biological activity of PGE2 (122). 11β-PGF2α was quantified in the cellular lysate and culture medium of control and LPS-exposed cells (**Figures 21** and **23**). The increase of PGE2 in the culture medium of LPS-stimulated macrophages compared to control group was accompanied by a decrease of 11β-PGF2α (**Figure 23**). PGD2 is a structural isomer of PGE2, and this can explain the opposite tendency observed for this prostanoid. In unstimulated macrophages PGD2 generation is coupled to the constitutive COX-1 (117). So, the decrease of 11β-PGF2α in the culture medium of LPS-stimulated cells when compared to control cells can be justified by the preferred production of PGE2, which is in agreement with bibliography (117). This fact can also explain why the extract had no effect in the release of 11β-PGF2α into the medium, since phenolic compounds mainly affect COX-2 (123). However, the intracellular level of 11β- PGF2α was increased by LPS and a decrease of this metabolite was observed when cells were also exposed to the extract (except for the highest concentration) in comparison with cells exposed only to LPS (**Figure 21**).

As previously said, the bee pollen extract used in this work was characterized by the presence of kaempferol and quercetin derivatives. It is well known that kaempferol inhibits iNOS, COX-2 expression and PGE2 formation in LPS-activated RAW macrophages (124). Also quercetin was found to decrease PGE2 in the culture medium of LPS-stimulated RAW macrophages (125), the reduction being much stronger in J774A.1 macrophages (126).

The iNOS inhibitory activity of flavonoids is attributed to the inhibition of NF-kB induction (124). The more active flavonoids possess a double bond between C-2 and C-3 and a 5,7-dihydroxyl group. The hydroxyl group at C-3 decreases the inhibitory potency (93). On the other hand, COX-2 inhibition by flavonoids is less studied, but is also partly attributed to NF-kB inhibition. NFkB is involved in COX-2 protein expression in LPS-stimulated J774.1 macrophages and the exposition of these cells to NF-kB inhibitors before LPS challenge abrogates the generation PGE2 and 6-keto PGF1α (125). In fact, 6-keto PGF1α release was inhibited by the extract, except for the lowest concentration tested, while a decrease of PGE2 release was only observed for this concentration (**Figure 23**).

It can be expected polyphenol aglycones to be more potent than their heterosides. For instance, Kim and collaborators showed that isoliquiritigenin was significantly more active than its 4-*O*-glucoside (isoliquiritin) in reducing •NO and PGE2 production, as well as protein and mRNA expression of iNOS and COX-2 (126). This result was explained by the different lipophilicity, which affects the cellular uptake and compatibility with cellular membranes (126). Also the inhibition of iNOS and COX-2 expression by quercetin was higher than the one of its 3-*O*-rutinoside, rutin (125). Nevertheless, the decrease of PG observed when cells were exposed to the extract at 0.5 or 0.1 mg mL^{-1} is probably due to the inhibitory action of the flavonol heterosides that characterize the bee pollen extract.

It has been suggested a cross-talk between NOS and COX enzymes (127). •NO may act through generation of peroxynitrite as the endogenous oxidant needed to create the tyrosyl radical required for catalytic activity of COX enzymes. •NO exerts divergent effects on constitutive and inducible COX isoforms, activating COX-1, but inactivating COX-2 (127). This may explain why extract concentrations that reduce •NO to lower levels failed to reduce the levels of PGE2, the major PG released by LPS stimulated macrophages (**Figures 21** and **23**).

Conversely, PGE2 demonstrated to modulate •NO pathway in LPS-stimulated

macrophages (128). These results were not confirmed by Swierkosz and collaborators, who found that the formation of COX metabolites had no effect on NOS activity, whereas $^{\bullet}NO$ inhibits both COX-2 activity and induction (129).

IsoPs are derived from the free radical-catalysed peroxidation of AA in a COX independent mechanism (**Figure 22**). Due to the lack of enzymatic control, a series of stereoisomers can be formed (122). IsoPs are reliable markers of lipid peroxidation *in vivo* and potentially mediate some of the adverse effects of oxidant injury (130). In this study three IsoPs of the 8-series (containing the nonprostane hydroxyl group at C-8) were encountered, both in the cell lysate and in the culture medium (**Figures 21** and **23**). In general, LPS treatment leads to an increase of IsoPS, which can be a consequence of the increase of $^{\bullet}NO$ in LPS-exposed macrophages. The excess of $^{\bullet}NO$ production can lead to peroxynitrite formation, which is associated with increased IsoP generation *in vitro* and *in vivo* (131, 132).

8-iso-PGF2α showed a different behaviour, since the release of this IsoP into the culture medium after 8 h exposition to LPS decreased when compared with control cells (**Figure 23**). 8-iso-PGF2α can be formed *via* COX-2 in monocytes and, as so, it was expected to increase under a pro-inflammatory stimulus (133). Nevertheless, at this time the level of 8-iso-PGF2α in cell lysate of LPS stimulated cells was higher than in control cells (**Figure 21**).

IsoPs levels showed a tendency to be increased by extract concentrations above 1 mg mL^{-1}, despite the decrease of $^{\bullet}NO$ contents (**Figures 20, 21** and **23**). This can be attributed to a pro-oxidant behaviour of phenolic compounds when present at high concentrations (observed in the $O_2^{\bullet-}$ cell-free assay), despite the ability of these compounds to inhibit iNOS induction in macrophages under an inflammatory stimulus (125, 126).

1.2.3. Effect on degranulation

In this study we used a crude extract to evaluate the anti-allergic potential of *E. plantagineum* bee pollen since bee pollen is consumed in folk and complementary medicines as a whole (134). So the extraction method used allowed the recovery of a wide variety of compounds, in order to guarantee that the compounds that have an activity *in vivo* were not lost during the extraction process.

The extract revealed to be composed by several organic and fatty acids, as shown in **tables 4** and **5**, respectively. These compounds could affect the results of degranulation

since degranulation phenomenon is influenced by pH (135). However, as the pH range of the medium used in the assays was buffered to pH 6.8–7.2, the amounts of acids in the extracts were not sufficient to exceed the buffering capacity of the culture medium and so degranulation was not affected.

1.2.3.1. Effect on cell viability

First, the effects of the hydromethanol extract on cellular viability were evaluated by the MTT reduction assay. As it can be seen in **Figure 24**, the extract was not cytotoxic to RBL-2H3 cells in the range of concentrations used to evaluate its anti-allergic potential (0.52 to 2.08 mg mL^{-1}). The extract by itself did not affect cell viability till the concentration of 8.33 mg mL^{-1} (data not shown). However, basophils pre-exposed to the hydromethanol extract for 15 min followed by co-exposition for 30 min with calcium ionophore (500 ng mL^{-1}) suffered a significant decrease of viability for concentrations above 4.17 mg mL^{-1} (data not shown). So, as ionophore A23187 is highly selective for calcium (136) it can be assumed that the interaction of the compounds present in the extract with the calcium ionophore leads to cellular calcium overload, compromising cellular viability for the higher extract concentrations tested.

MTT reduction assay was also performed for RBL-2H3 cells treated with IgE/antigen in the presence and in the absence of the extract. The IgE/antigen treatment by itself did not affect basophils viability, either in the presence or absence of the extract (**Figure 24B**).

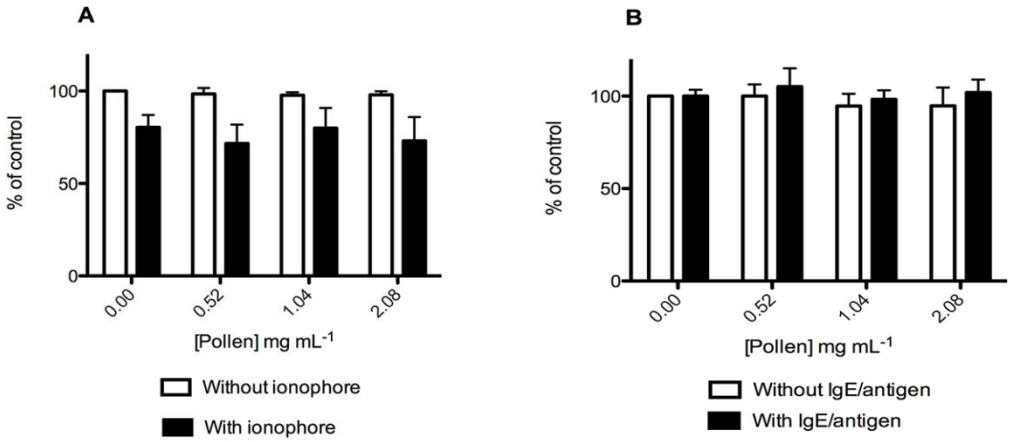


Figure 24. Effect of *E. plantagineum* bee pollen extract in RBL-2H3 basophils viability, assessed by MTT reduction assay. Values show mean ± SEM of three independent assays performed in duplicate with calcium ionophore (or extract only) and six independent assays performed in duplicate with IgE-DNP – DNP-BSA (or extract only). (**A**) Cells were pre-exposed to the extract for 15 min, followed by 30 min co-exposure to 500 ng mL^{-1} calcium ionophore A23187; (**B**) Cells were pre-exposed to 100 ng mL^{-1} anti-DNP IgE for 16 h, followed by 1 h extract co-exposure to 100 ng mL^{-1} DNP-BSA.

1.2.3.2. Effect on β-hexosaminidase release

The release of β-hexosaminidase enzyme, a marker of degranulation in mast cells and basophils, allows estimating the anti-allergic potential of new drugs *in vitro* (83). In a first approach we used calcium ionophore A23187 to elicit degranulation of RBL-2H3 cells. Artificial activation of basophils by calcium ionophore A23187 is routinely used in the research of exocytosis to bypass the molecular events that are associated with the excitation of FcεRI receptors. With this stimulus the extract inhibited β-hexosaminidase release in a dose-dependent manner (p<0.001) (**Figure 25A**). Additionally, β-hexosaminidase release was further investigated using higher extract concentrations. It was observed that the extract decreased β-hexosaminidase release at 4.17 mg mL^{-1} (64.42% ± 2.55%, p<0.001), but at this concentration the cellular viability was significantly affected (data not shown). The subsequent increase of β-hexosaminidase release at 8.33 mg mL^{-1} (90.98% ± 6.58%) may result not only from cell degranulation, but also from enzyme leakage by unviable cells with disrupted membrane integrity (137).

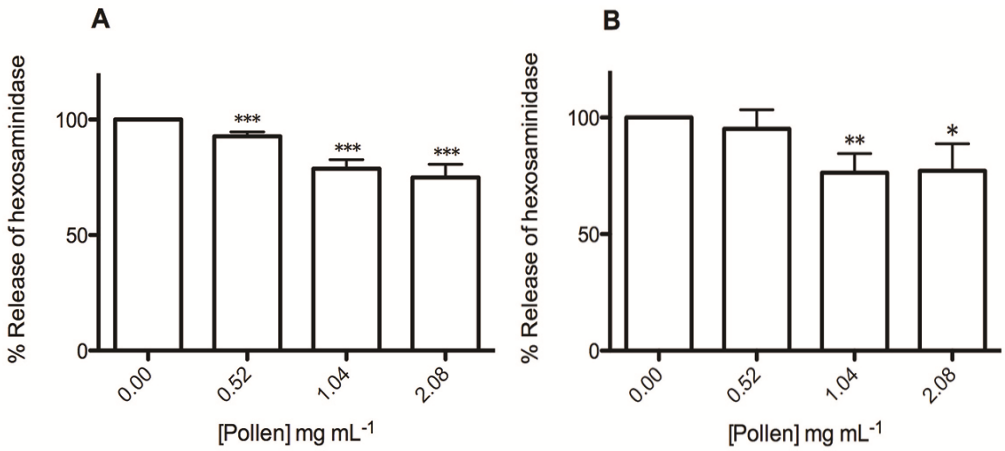


Figure 25. Effect of *E. plantagineum* bee pollen extract in β-hexosaminidase release. Values show mean ± SEM of three independent assays performed in duplicate with calcium ionophore stimulus and six independent assays performed in duplicate using IgE-DNP – DNP-BSA stimulus. (**A**) % of β-hexosaminidase release after RBL-2H3 exposure to ionophore; *** p<0.001. (**B**) % of β-hexosaminidase release after RBL-2H3 pre-exposure to anti-DNP IgE, followed by DNP-BSA exposure; * p<0.05; ** p<0.01.

The anti-allergic potential of the extract was also evaluated using IgE/antigen stimulus, since basophils possess high-affinity IgE receptors. As referred above, the cross-linking of FcεRI-bound IgE by allergens plays a pivotal role in IgE-mediated allergy (138). Using this allergic stimulus the co-exposition with the extract significantly inhibited the DNP-BSA-induced β-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells at 1.04 and 2.08 mg mL^{-1} (23.61% ± 8.15%, p<0.01, and 22.84% ± 11.57%, p<0.05, respectively) (**Figure**

25B). As observed with the calcium ionophore, when higher concentrations of the extract were assayed, an increase in β-hexosaminidase release was observed (data not shown). However, with this stimulus there was no observable decrease of cell viability. So, we can argue that at higher concentrations the extract demonstrated a potential allergic effect in IgE sensitized basophils. In fact, the hydromethanol extract has demonstrated a pro-oxidant potential at these same concentrations, as mentioned in section **1.2.1.** .Thus, it may be speculated that the generation of reactive oxygen species influenced the signaling pathways evoked by the IgE/antigen stimulus, leading to enhanced degranulation. This behaviour has already been observed for compounds with pro-oxidant activity (139).

The anti-allergic potential demonstrated by the extract can be attributed to several compounds, namely to the polyphenols shown in **table 3** and to the fatty acids displayed in **table 5**. Palmitic and α-linolenic acids, the two major fatty acids identified in the extract, have known anti-allergic effects. In fact, among the fatty acids found in the extract, α-linolenic acid has been demonstrated to reduce the release of inflammatory mediators, including histamine and prostaglandin E2 (140). Moreover, epidemiological studies show that high intake of α-linolenic and palmitic acids during pregnancy may decrease the risk of asthma of the offspring (141). So, the anti-degranulation effects observed in the model used in the present study can result partially from the presence of fatty acids in the extract.

Concerning to polyphenols, these compounds are well known for their anti-allergic potential (141). In fact, the tested extract contains derivatives of quercetin and kaempferol (**Table 3**), which have inhibitory effect on degranulation of mast cells and basophils (142). This effect is thought to result from two mechanisms: one by which polyphenols impact allergen-IgE complex formation, and another through which they impact on the binding of this complex to their basophil FcεRI (142). This can be explained by the formation of insoluble complexes between the polyphenols and potential allergenic proteins, rendering them to be hypoallergenic (142). Nevertheless, the extract was more effective in inhibiting degranulation when the calcium ionophore was used: the effective concentrations ranged between 0.52 mg mL^{-1} and 2.08 mg mL^{-1}, while in the assay with IgE/antigen the effective concentrations varied between 1.04 mg mL^{-1} and 2.08 mg mL^{-1}, suggesting that the compounds present in the extract also affected the increase of cytoplasmic calcium, an event that immediately precedes degranulation.

A direct effect of organic acids in degranulation could not be established. However, as malonic and acetic acids are precursors of fatty acids and also of flavonoids (together with shikimic acid), the determination of organic acids was considered important for the

characterization of the metabolic profile of this matrix.

In order to clarify whether the effects of the extract were due to the inhibition of hexosaminidase release, and not a false positive resulting from the inhibition of β-hexosaminidase activity, we tested the effect of the extract on β-hexosaminidase activity using a basophil cell lysate as source of β-hexosaminidase. As the extract did not inhibit β-hexosaminidase enzymatic activity (data not shown), the β-hexosaminidase results observed in basophils insulted with an allergic stimulus are due to the extract effects on degranulation.

Considering the results obtained with the extract, although the percentage of inhibition of degranulation was not very high, it was significantly different from the control, justifying the traditional use of bee pollen in allergic diseases.

1.2.3.3. Effect on soybean lipoxygenase activity

Lipoxygenases are enzymes involved in the AA pathway, which catalyze the peroxidation of polyunsaturated fatty acids in a selective way. 5-Lipoxygenase metabolizes free AA to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE), which is further metabolized to leukotrienes. These compounds are pivotal lipid mediators in allergy and inflammation (143). Because of structural and functional similarities with mammalian enzymes, lipoxygenase obtained from soybean is widely accepted as a model for lipoxygenase inhibition studies (144). In this assay, *E. plantagineum* bee pollen hydromethanolic extract was not able to inhibit the activity of this enzyme. As shown in section **1.2.2.3.**, the extract decreased the levels of AA metabolites derived from COX-2 in LPS-stimulated macrophages, demonstrating that it has anti-inflammatory activity. As during an allergic reaction an inflammatory activity is involved, the extract can contribute to ameliorate the symptoms of allergy, through a mechanism not involving the inhibition of lipoxygenase.

1.2.4. Antibacterial capacity

In order to evaluate the antimicrobial potential of *E. plantagineum* bee pollen, the hydro- methanol extract was screened against Gram-positive and Gram-negative bacteria.

Concerning to the effect against Gram-positive bacteria, *S. aureus* was the only one for which it was possible to determine the MIC value (**Table 6**). The extract also showed

some effect against other two Gram-positive bacteria, *S. epidermidis* and *M. luteus*, with MLC values of 100 and 200 mg mL^{-1}, respectively (**Table 6**).

Table 6. MIC and MLC values (mg mL^{-1} pollen) obtained with *E. plantagineum* bee pollen hydromethanol extract against selected bacteria.

Microorganism	MIC	MLC
Gram-positive		
S. aureus	200	100
S. epidermidis	-	100
M. luteus	-	200
E. faecalis	-	-
B. cereus	-	-
Gram-negative		
P. mirabillis	-	-
E. coli	-	-
P. aeruginosa	-	-
S. typhimurium	-	-

Under the tested concentrations, all Gram-negative bacteria were found to be resistant to the extract. Previous studies indicated that Gram-negative bacteria appear to be less sensitive to the action of several natural extracts (114), which agrees with the results above presented. The reason for this may be related with the physical differences between the cell walls of Gram-positive and Gram-negative bacteria. The external membrane with high content of lipopolysaccharide that surrounds the cell wall in the latter is considered to be the major factor contributing to its resistance to chemical stress (115, 116).

From the obtained data it can be stated that *E. plantagineum* bee pollen has low anti-bacterial activity and only in very high concentrations. As the bee pollen contains organic acids, the activity observed might be attributed to these compounds, as they have been reported to exhibit general antimicrobial activities and are commonly used as food preservatives. Also, it is known that plants synthesize phenolic compounds in response to

microbial infection (117) and have shown antimicrobial activity *in vitro* against a wide range of microorganisms. Phenolic activity is concentration-dependent and possibly its concentration in the extract was not sufficiently high as to cause a marked bacterial inhibition.

2. Acidified methanol extract

2.1. Phenolic composition

In order to study the protective effect of *E. plantagineum* bee pollen on cells of the digestive tract, an acidified methanol extract was prepared. The purpose was to assess the effect of each of the two classes of phenolic compounds present in the extract, non-coloured flavonoids and anthocyanins, and also of the extract as a whole. As anthocyanins are positively charged at acidic pH and several authors reported that acidified methanol preserves the extracted anthocyanin in their original form, better than in a non-acidified solvent system (145), the methanol extract was acidified. The crude extract was fractioned by SPE using ethyl acetate and methanol as eluents. Two fractions were separated, one enriched in non-coloured flavonoids (fraction I) and the other enriched in anthocyanins (fraction II). Twelve phenolic compounds were identified, belonging to flavonols (7 compounds) and anthocyanins (5 compounds), as described below.

2.1.1. Non-coloured phenolic profile

2.1.1.1. Qualitative analysis

The determined non-coloured phenolic compounds were all derivatives of kaempferol (**Table 7**, **Figure 26**). Fraction I (eluted with ethyl acetate) contained six compounds, while fraction II (collected with methanol, after ethyl acetate elution) and the whole extract contained all the identified compounds. In all of the cases the major compound found was kaempferol-3-*O*-neohesperidoside, representing between 73.2% (fraction I) and 75.2% (whole extract) of the identified compounds.

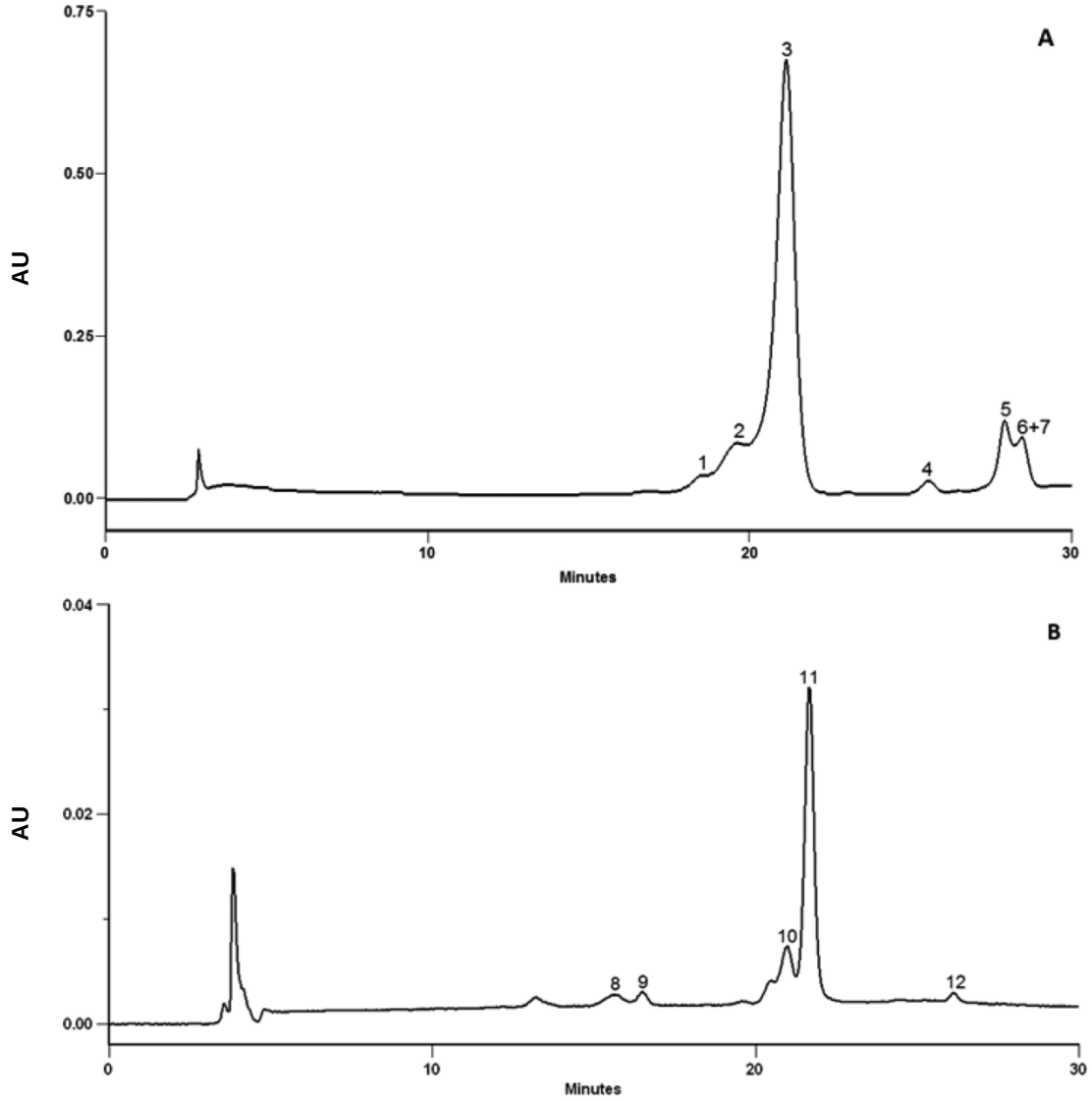


Figure 26. HPLC-DAD chromatograms of *E. plantag*ineum bee pollen: (**A**) non-coloured phenolic compounds in the whole extract (350 nm); (**B**) anthocyanins in fraction II (500 nm). Compounds: (**1**) kaempferol-3-*O*-(4''-rhamnosyl)-neohesperidoside; (**2**) kaempferol-3-*O*-sophoroside; (**3**) kaempferol-3-*O*-neohesperidoside; (**4**) kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside; (**5**) kaempferol-3-*O*-glucoside; (**6+7**) kaempferol-3-*O*-rutinoside + kaempferol-3-*O*-(3''/4''-acetyl)-neohesperidoside isomer. Compounds: (**8**) delphinidin-3-*O*-glucoside; (**9**) delphinidin-3-*O*-rutinoside; (**10**) petunidin-3-*O*-glucoside; (**11**) petunidin-3-*O*-rutinoside; (**12**) malvidin-3-*O*-rutinoside.

2.1.1.2. Quantitative analysis

The total amount of flavonols was 5.6 times higher in fraction I than in fraction II, the amount in the whole extract approximately corresponding to the sum of the flavonols present in both fractions (6.5 ± 2.0 g kg^{-1}) (**Table 7**). Fraction I contained higher relative amounts of compounds **2**, **5** and **6 + 7** than fraction II, while compound **1** was absent. No

statistical differences were found for the relative amounts of compounds **3** and **4** (p>0.05).

Table 7. Non-coloured phenolic compounds in *E. plantagineum* bee pollen acidified methanol extract and fractions (mg Kg^{-1} pollen)[1].

Compounds		Fraction I	Fraction II	Whole
1	Kaempferol-3-*O*-(4''-rhamnosyl)-neohesperidoside	-[a]	148.27 ± 6.68[b]	146.07 ± 23.91[b]
2	Kaempferol-3-*O*-sophoroside	658.49 ± 71.15[a]	87.34 ± 10.20[b]	635.68 ± 63.57[a]
3	Kaempferol-3-*O*-neohesperidoside	4094.46 ± 49.52[a]	735.28 ± 19.08[b]	4890.54 ± 118.13[c]
4	Kaempferol-3-*O*-neohesperidoside-7-*O*-rhamnoside	70.16 ± 0.35[a,b]	8.31 ± 0.55[a]	91.74 ± 1.64[b]
5	Kaempferol-3-*O*-glucoside	395.47 ± 21.83[a]	10.82 ± 1.44[b]	402.17 ± 3.76[a]
6 + 7	Kaempferol-3-*O*-rutinoside + kaempferol-3-*O*-(3''/4''-acetyl)-neohesperidoside isomer	373.75 ± 8.53[a]	7.40 ± 0.65[b]	336.30 ± 14.58[a]
	Total	**5592.33 ± 151.38[a]**	**997.42 ± 31.93[b]**	**6502.50 ± 201.67[c]**

[1] Values are expressed as mean ± standard deviation of three determinations. Different superscript letters in the same row mean statistically significant differences (p<0.05 or higher).

2.1.2. Anthocyanins profile

2.1.2.1. Qualitative analysis

As referred in section **2.2.1.** of Introduction, the dark blue colour of *E. plantagineum* bee pollen is attributed to its anthocyanin content (37). Anthocyanins are soluble in polar solvents, but are unstable in neutral or alkaline media, being usually extracted from plant materials with acidified methanol to prevent alteration of acylated compounds (8). Using SPE, a fraction containing almost all the anthocyanins present in the acidified methanol extract was obtained by elution with methanol (fraction II), after eluting with ethyl acetate to remove some non-coloured phenolic compounds (100). The anthocyanins qualitative profile of fraction II can be seen in **Figure 26B**, being similar to the profile of the whole extract (not shown). In order to identify the compounds, an acidic hydrolysis was performed and the aglycones obtained were identified by comparison with authentic standards. The complete identification of the heterosides present in the extracts was done by comparison with standards and bibliography (37).

The chromatogram from fraction II of the acidified methanolic extract showed petunidin-3-*O*-rutinoside (**11**) as the major peak, followed by petunidin-3-*O*-glucoside (**10**) (**Figure**

26B).

2.1.2.2. Quantitative analysis

The total amount of anthocyanins was higher in the whole extract than in fraction II. Petunidin-3-*O*-rutinoside (compound **11**) was the major compound in both, being the only anthocyanin present in fraction I. This compound was found in the whole extract at higher quantity than its sum in fractions I and II. Delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside were present in higher concentration in fraction II than in the whole extract (**Table 8**). Because compound **11** co-eluted with compound **3**, the latter being more concentrated than the former in the whole extract, the quantification of compound **11** was less accurate in the whole extract (though the compounds absorb at different wavelengths). The total amount of identified anthocyanins corresponded to 0.313 mg kg^{-1} in fraction I, 32.6 mg kg^{-1} in fraction II and 38.3 mg kg^{-1} in the whole extract, compound **11** accounting for 100.0, 74.7 and 77.3% of the total identified anthocyanins, respectively.

Table 8. Coloured phenolic compounds from *E. plantagineum* bee pollen acidified methanol extract and its fractions (mg Kg^{-1} pollen)[1].

Compounds		Fraction I	Fraction II	Whole
8	Delphinidin-3-*O*-glucoside	-[a]	1.21 ± 0.05[b]	1.03 ± 0.03[b]
9	Delphinidin-3-*O*-rutinoside	-[a]	0.95 ± 0.06[b]	0.88 ± 0.10[b]
10	Petunidin-3-*O*-glucoside	-[a]	5.41 ± 0.30[b]	5.88 ± 0.48[b]
11	Petunidin-3-*O*-rutinoside	0.31 ± 0.003[a]	24.30 ± 0.17[b]	29.56 ± 0.58[c]
12	Malvidin-3-*O*-rutinoside	-[a]	0.68 ± 0.02[a,b]	0.92 ± 0.06[b]
	Total	**0.31 ± 0.003[a]**	**32.56 ± 0.60[b]**	**38.26 ± 1.26[c]**

[1]Values are expressed as mean ± standard deviation of three determinations. Different superscript letters in the same row mean statistically significant differences (p<0.05 or higher).

2.2. Biological effects

Protective effects were assessed in Caco-2 cells that mimic the enterocytes of small intestine.

2.2.1. Cell viability

The effect of *E. plantagineum* bee pollen acidified methanol extract on the viability of Caco-2 cells under quiescent conditions was evaluated by LDH leakage and MTT reduction assays (**Figure 27**). No significant toxicity was observed in the concentrations range from 0.31 to 20.0 mg mL^{-1} for fractions I and II. However, at the higher concentrations tested, the whole extract caused a significant decrease in cellular viability, as assessed by the MTT assay (p<0.0001, n=5). This loss of cellular viability cannot be attributed to one class of phenolic compounds, since neither fraction I (nearly free of anthocyanins), nor fraction II (containing 15% of total flavonols and anthocyanins), caused a significant decrease of cell viability at this concentration. Rather the combined effects of anthocyanins with high amounts of flavonols may be responsible for the observed toxicity.

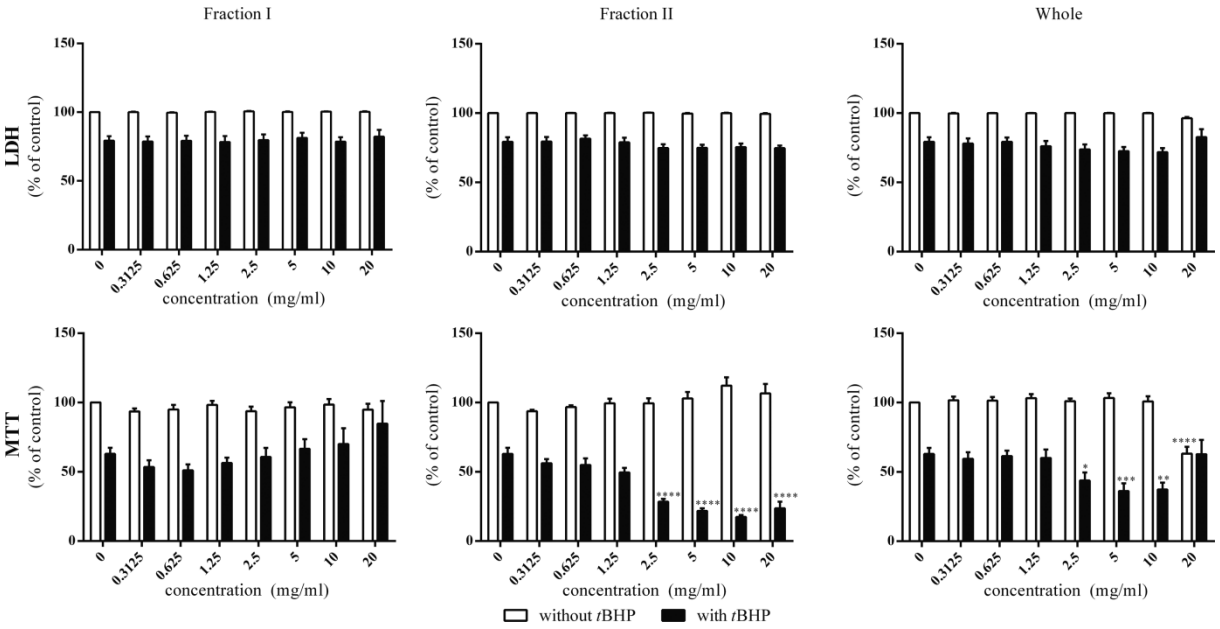


Figure 27. Viability of Caco-2 cells evaluated by LDH and MTT assays. Cells were treated with *E. plantagineum* bee pollen acidified methanol extract and fractions for 24 h, or were pre-treated for 24 h with *E. plantagineum* bee pollen acidified methanol extract and fractions followed by 6 h treatment with 150 µM of *t*-BHP (without extract co-incubation). Results are presented as mean ± standard error of the mean of five independent experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to the respective control (with or without *t*-BHP).

Flavonoids can act as antioxidants in their reduced form but, when oxidized to phenoxyl radicals or quinone/quinone methide intermediates, they can have pro-oxidant activities (146). A pro-oxidant effect has been described for both classes of flavonoids

present in *E. plantagineum* bee pollen. Flavonol compounds with a phenolic B ring like the kaempferol derivatives found in the extracts may act as pro-oxidants after oxidation by peroxidases/H_2O_2 to phenoxyl radicals, accompanied by GSH co-oxidation and oxygen activation, resulting in superoxide radical and H_2O_2 formation (147). Also, a pro-oxidant effect of anthocyanidins in HL-60 cells was attributed to intracellular hydrogen peroxide production, triggering an apoptotic death program through oxidative stress (148). This may explain why only in the MTT assay a significant loss of viability was observed.

Oxidative stress can be induced in cells by *t*-BHP, which is metabolized by Cyt P450, leading to the formation of toxic peroxyl and alkoxyl radicals that initiate lipid peroxidation, affect cell integrity and form covalent bonds with cellular molecules, resulting in cell death (149, 150). The exposition of Caco-2 cells to 150 µM *t*-BHP for 6 h lead to a reduction of viability to 79.3 ± 3.2 % of control in LDH leakage assay (p<0.0001, n = 5) and 63.0 ± 0.3 % in the MTT assay (p<0.001; n = 5). The higher sensitivity of MTT assay in detecting *t*-BHP toxicity can be explained by the fact that its biotransformation by Cyt P450 and GPx occurs mainly in the mitochondria (149, 151, 152). The pre-treatment of Caco-2 cells with the extracts did not prevented, nor aggravated, the toxicity of *t*-BHP, as measured by the LDH leakage assay (**Figure 27**). However, the extracts containing anthocyanins (fraction II and the whole extract) significantly aggravated *t*-BHP toxicity, as measured by the MTT assay, fraction II being more deleterious (**Figure 27**). When the cells were pre-treated with this fraction, a significant increase of cell death (compared to the control with *t*-BHP) was observed for extract concentrations higher than 1.25 mg mL^{-1} (p<0.0001, n=5), while with the whole extract the differences in cellular viability were only seen between 2.5 and 10 mg mL^{-1} (p<0.001 or higher, n=5). The additional cellular death, observed only in the MTT assay, may be a consequence of the depletion of cellular defences of cells pre-exposed to anthocyanins, due to their ability to induce the production of reactive species (148).

On the other hand, fraction I showed a tendency to protect Caco-2 cells from *t*-BHP-induced toxicity, directly dependent on its concentration, although the differences of cellular viability were not statistically significant (**Figure 27**). The viability of cells pre-treated with faction I at the concentration of 20 mg mL^{-1} was increased to 84.7 ± 16.3% of control, as measured by the MTT assay. Also, the pre-exposition to the whole extract at 20.0 mg mL^{-1} did not increased cellular death assessed by the MTT assay (p>0.05, n=5), which was nearly the same has in the cells exposed to *t*-BHP only, although at this concentration the whole extract induced cellular death by itself (**Figure 27**). It can be assumed that, in insulted cells, the pre-exposition to flavonols may stimulate antioxidant defences, which help fighting the subsequent oxidative stress induced by *t*-BHP. Probably, the pro-oxidant effects of anthocyanins do not prepare the cells for a

subsequent insult, resulting in increased toxicity. As observed in the assay with the whole extract at 20 mg mL^{-1}, this toxicity can be counteracted by the defences induced by flavonols, although they contribute to the overall oxidative stress observed in cells exposed to the extract.

The effects of *E. plantagineum* bee pollen in cellular viability greatly depend upon the cell line. In fact, as shown above, the hydromethanol extract of this material caused loss of macrophages viability at concentrations higher than 30 mg mL^{-1} (**Figure 19**), but basophils revealed decreased viability at 8 mg mL^{-1} (section **1.2.3.1.**). Since macrophages are known to produce reactive species (153) and the gastrointestinal tract is constantly exposed to reactive oxygen, chlorine, and nitrogen species (154), the sensitivity of the different cells lines can be explained by the oxidative stress induced by extract compounds and cellular ability to oppose to it.

2.2.2. Reactive species

The levels of reactive species were measured by the DCFA-DA assay. The exposure of Caco-2 cells to 150 µM *t*-BHP lead to a significant increase of reactive species to 126.5 ± 1.4% of control after 30 min ($p<0.0001$, n = 5), reaching a maximum of 133.7 ± 1.8% after 150 min, and decreasing to 130.1 ± 2.9% after 240 min. Fraction I significantly decreased the levels of reactive species in a concentration and time dependent manner, this ability being reduced over time (**Figure 28**). The lower concentrations of fraction I and of the other extracts were more effective in reducing reactive species (**Figure 28**). For instance, pre-exposure to 1.25 mg mL^{-1} of fraction I reduced the level of reactive species induced by *t*-BHP to 111.6 ± 3.6% after 30 min ($p<0.01$), but after 240 min the level of reactive species achieved was 116.6 ± 4.2% ($p<0.05$). On the other hand, the levels of reactive species were 122.4 ± 2.0% ($p>0.05$) after 30 min and 137.7 ± 5.4% ($p>0^{-1}.05$) after 240 min, when the cells were pre-exposed to the extract at 20 mg mL^{-1}.

In cells pre-exposed to fraction II a more pronounced effect on the levels of reactive species induced by *t*-BHP was observed (**Figure 28**). Excepting 20 mg mL^{-1}, all concentrations lead to a significant decrease in the levels of reactive species during the 240 min evaluation period ($p<0.01$ or higher). It should be noted that the levels of reactive species were reduced to the levels attained in cells not exposed to *t*-BHP when the cells were pre-exposed to 1.25 mg mL^{-1} of fraction II (100.6 ± 4.5% after 30 min and 99.1 ± 3.8% after 240 min exposition to *t*-BHP). Although no statistical difference was seen for the concentration of 20 mg mL^{-1}, the levels of reactive species were lower than in cells not

pre-treated with extract (116.9 ± 3.5 mg mL^{-1} after 30 min and 129.0 ± 3.7 mg mL^{-1} after 240 min).

Surprisingly, the whole extract, containing the compounds of both fractions, not only was unable to reduce the levels of reactive species induced by *t*-BHP, but further aggravated the oxidative stress after 180 min in cells pre-exposed to 20 mg mL^{-1} of the extract (**Figure 28**). At this concentration, the levels of reactive species reached 152.4 ± 5.9% after 240 min exposition to *t*-BHP ($p<0.0001$). However, at the beginning of the measurements they were of 115.2 ± 3.6% ($p>0.05$), and for lower concentrations no statistical differences were seen (116.8 ± 1.5% after 30 min and 126.7 ± 1.7% after 240 min in cells pre-exposed to 1.25 mg mL^{-1} of whole extract). The whole extract was the one with higher variations in the levels of reactive species measured over time, being this effect detected earlier with higher concentrations. It showed a tendency to increase the levels of reactive species over time, with statistical differences being noticed, from the concentration of 2.5 mg mL^{-1}, while with the other extracts a significant increase was only seen at 20 mg mL^{-1} for fraction II and 10 mg mL^{-1} for fraction I.

Although the levels of reactive species were measured without taking into account the viability of cells, a significant decrease in this parameter measured by the MTT assay was seen after 6 h exposure to *t*-BHP in cells pre-exposed to concentrations from 2.5 mg mL^{-1} of fraction II and from 2.5 to 10 mg mL^{-1} of the whole extract (**Figure 28**). So, the results may reflect the levels of reactive species in viable and unviable cells.

Fraction II, which showed the higher ability to aggravate the toxicity induced by *t*-BHP, was the one that reduced the levels of reactive species more pronouncedly (**Figure 28**). It is possible that reactive species produced during the pre-exposition to anthocyanins and other compounds present in fraction II have inactivated Cyt P450, diminishing in this way the biotransformation of *t*-BHP and, consequently, the levels of reactive species. The inhibition of the activity of several Cyt P450 isoforms by anthocyanins has already been described (155, 156). For instance, petunidin inhibited Cyt P450 3A4 activity with an IC_{50} of 23 µM (155). Flavonols (present in both fractions, as well as in the whole extract) may also contribute to the inhibitory activity of these enzymes (157). The viability of cells pre-exposed to higher concentrations of fraction II was adversely affected, as seen by the MTT assay (**Figure 27**), and the levels of reactive species induced by *t*-BHP were higher than in cells exposed to lower extract concentrations (**Figure 28**). It is possible that when cells with lower Cyt P450 activity were exposed to *t*-BHP, new radical species were generated by non-enzymatic reaction of *t*-BHP with iron, as demonstrated by Castilho and collaborators in an experiment using the iron chelator *o*-phenantroline with diminished toxicity of *t*-BHP (158). This fact may also explain the increased toxicity observed with the

whole extract, which can be due to the combined effects of anthocyanins and high amounts of flavonols in Cyt P450 inhibition.

In addition to Cyt P450 inhibition, the antioxidant ability of fractions I and II seen in this assay can be attributed to direct scavenging effect or metal chelating properties of the flavonoids absorbed by the cells previously to *t*-BHP exposure (159, 160).

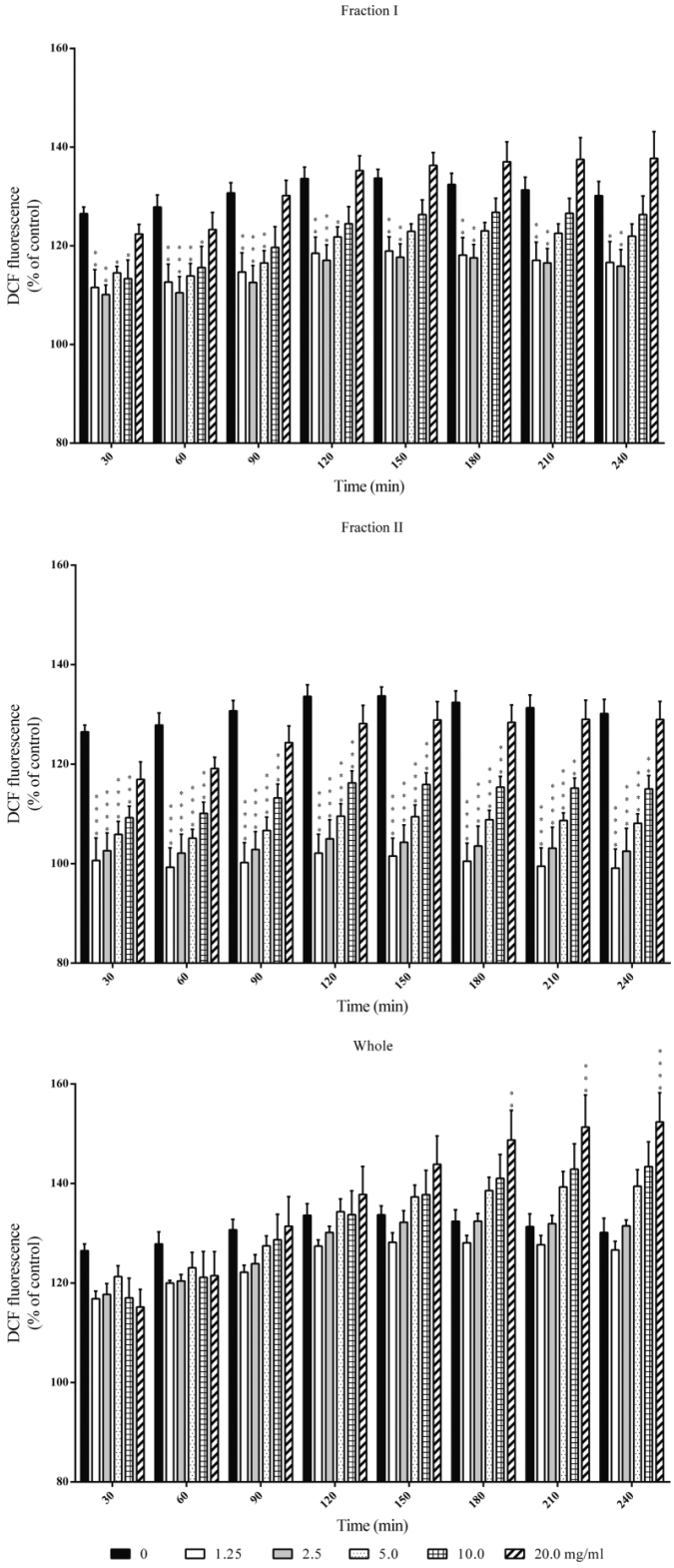


Figure 28. Reactive species in Caco-2 cells exposed to 150 µM *t*-BHP after pre-treatment with *E. plantagineum* bee pollen acidified methanol extract and fractions for 24 h. Results are presented as mean ± standard error of the mean of five independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 compared to the control (with *t*-BHP).

2.2.3. Glutathione homeostasis

The involvement of GSH in *t*-BHP detoxification is well known (161). *t*-BHP acts as a substrate for cytosolic glutathione peroxidases, causing the oxidation of GSH to glutathione disulphide (GSSG). The activity of GR operating at the expense of NADPH and the release of GSSG into the extracellular medium maintains a steady state in intracellular levels of GSH (162). In this work, the levels of glutathione (reduced + oxidized; GSHt) were of 22.88 ± 1.72 nmol mg^{-1} protein in quiescent cells and significantly increased to 42.84 ± 3.12 nmol mg^{-1} protein after 6 h exposure to 150 µM *t*-BHP (p<0.01, n=5). A further increase of GSHt levels was observed in cells previously exposed to 20 mg mL^{-1} of the whole extract (p<0.05, n=5) (**Figure 29**). It has been reported that the exposure of isolated hepatocytes to *t*-BHP leads to a rapid decrease of intracellular GSH levels (150, 163). However, similarly to the results found in this work, in Caco-2 cells an increase in GSH levels exposed to *t*-BHP has been already observed (164). The results may suggest an attempt of the cells to overcome the induced oxidative stress, by increasing GSH, the main antioxidant involved in *t*-BHP detoxification (161).

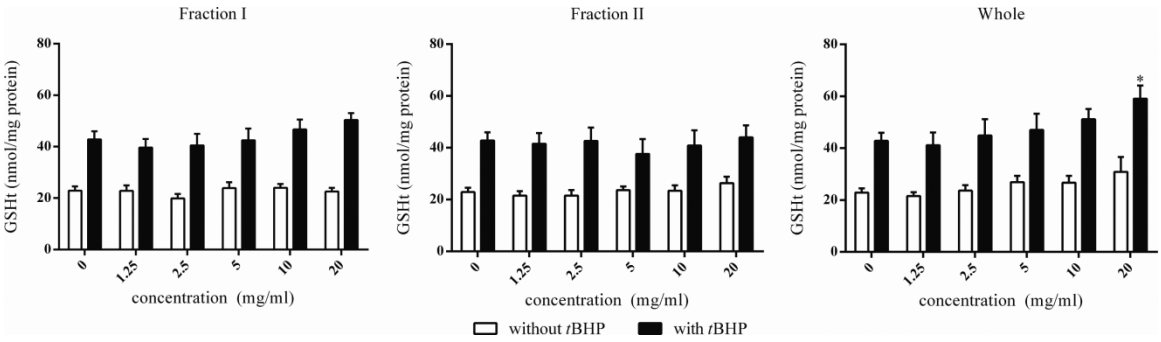


Figure 29. Glutathione levels in Caco-2 cells treated with *E. plantagineum* bee pollen acidified methanol extract and fractions for 24 h, or pre-treated for 24 h with *E. plantagineum* bee pollen acidified methanol extract and fractions followed by 6 h treatment with 150 µM of *t*-BHP (without extract co-incubation). Results are presented as mean ± standard error of the mean of five independent experiments. *p<0.05, compared to the respective control.

The increase of GSHt with the higher concentration of the whole extract (and less significantly with the higher concentration of fraction II and with 5 mg mL^{-1} of fraction I) may justify an inversion in the tendency of the extracts to aggravate the deleterious effects of *t*-BHP, although not accompanied by a significant reduction of the levels of reactive species seen with lower extract concentrations (**Figure 30**). The balance between antioxidant and pro-oxidant effects of the compounds present in the extract and fractions may justify their dual behaviour. It should be noted that the effects of flavonoids are not

limited to antioxidant or pro-oxidant mechanisms, since these compounds can interfere with many cellular molecules and processes (8, 146).

Under the assay conditions it was not possible to determine the intracellular levels of GSSG (below 0.38 nmol mg^{-1} protein). The low levels of GSSG (less than 1% of GSHt) can be explained by both its reduction to GSH by GR and its efflux from the cell (162).

2.2.4. Antioxidant enzymes

Since *t*-BHP detoxification involves its reduction by glutathione peroxidase (GPx) with concomitant oxidation of GSH to GSSG (162), an attempt was made to quantify GPx and GR, the later implicated in the recycling of GSSG back to GSH. Under the assay conditions, the activity of GPx was below the detection limit of the method (p<0.02 U mg^{-1} protein). GR activity in cells exposed to the extract or fractions for 24 h was not statistically different from control (0.027 ± 0.001 U mg^{-1} protein) at the concentrations tested (p>0.05; n=5).

Mitochondrial GSH transferases (GST) may prevent lipid peroxidation in mitochondria by a non-selenium glutathione-dependent peroxidase activity. Because *t*-BHP is a known substrate for GST, the activity of this enzyme was determined, being of 0.101 ± 0.006 U mg^{-1} protein in control cells. GST activity in cells exposed to the extracts was not statistically different from the control (p>0.05; n=5). However, the non-coloured phenolic fraction showed a tendency to decrease GST activity, reaching 0.089 ± 0.006 U mg^{-1} protein at the concentration of 20 mg mL^{-1}.

Although *t*-BHP does not react with catalase (165), the activity of this enzyme and of SOD was evaluated, due to their importance in the detoxification of H_2O_2 and superoxide that can be produced by a pro-oxidant effect of flavonoids, including flavonols and anthocyanins (147, 148), and in the maintenance of the redox cellular status, in general. Catalase activity in control was 7.444 ± 1.012 U mg^{-1} protein. When the cells were exposed to *E. plantagineum* bee pollen extract or fractions the enzyme activity varied between 5.998 ± 0.647 and 6.839 ± 0.829 U mg^{-1} protein. No statistical differences were seen for catalase activity (p>0.05; n = 5).

The extracellular SOD is a copper-zinc isoform of SOD that contains a signal peptide that directs this isoform exclusively to extracellular spaces (166). The activity of this enzyme in control was 0.100 ± 0.009 U mg^{-1} protein. Because the extracts exert a direct activity on superoxide, it was not possible to distinguish between the effects on SOD

activity and the superoxide scavenging activity of the extracts. For this reason an assay was performed using the extract and fractions incubated in culture medium, under the same conditions used to evaluate SOD activity, but without cells.

The results obtained for the variation of superoxide with or without the cells are presented in **Figure 30**. Fraction I was able to decrease superoxide levels, but this ability was higher in the cell-free assay, indicating a scavenging activity rather than induction of extracellular SOD. Significant differences from the control were observed from 2.5 mg mL^{-1} (p<0.01; n=4) in the cell-free assay, and from 10 mg mL^{-1} (p<0.05, n=5) in the assay with cells. Comparing the two assays (with and without cells), they were significantly different from 5.0 mg mL^{-1} (p<0.0001). This result can be explained by the inhibition of extracellular SOD, but, it is possible that some compounds were absorbed or modified by the cells, and were not available for superoxide scavenging. Considering fraction II and the whole extract, they exerted a slight scavenging activity (not statistically significant) for concentrations up to 5 mg mL^{-1} in the cell-free assay. This tendency was not observed in the assay with cells. At the concentration of 20 mg mL^{-1} a pro-oxidant effect was observed in both assays, although it was statistically significant only with fraction II in the assay with cells (p<0.001).

The ability of fraction I to scavenge superoxide radicals may contribute to the tendency of this extract to protect Caco-2 cells from *t*-BHP induced toxicity (**Figures 28 and 30**).

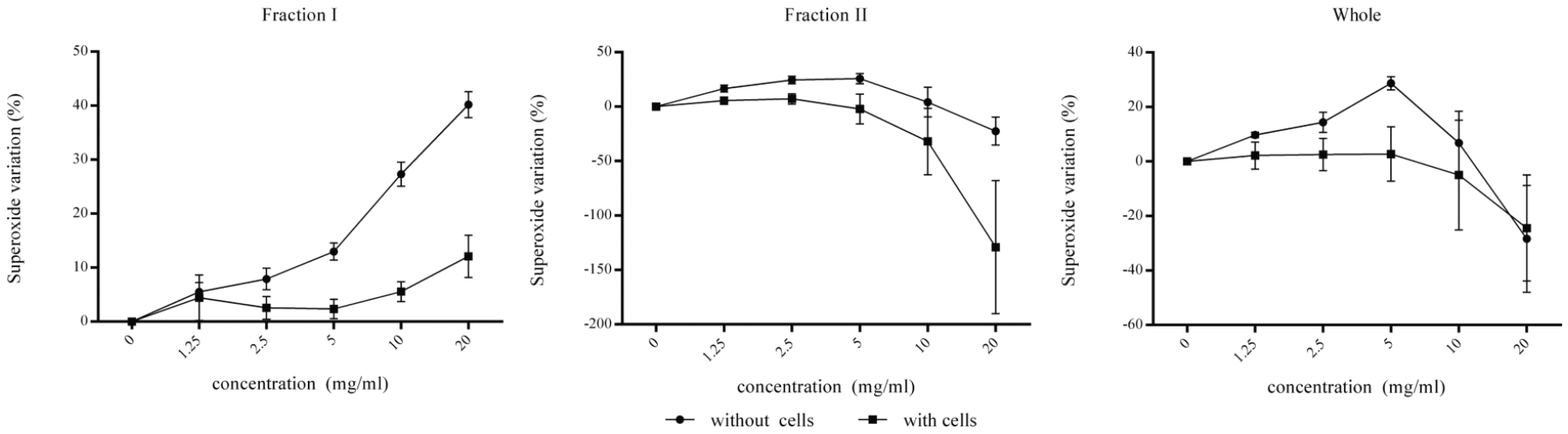


Figure 30. Effect of *E. plantagineum* bee pollen acidified methanol extract and fractions in superoxide levels. Superoxide was generated by the PMS/NADH system, and monitored by the reduction of NBT, in the presence of the extracts dissolved in culture medium and previously incubated for 24 h in a CO_2 incubator at 37 °C either with or without cells. Results are presented as mean ± standard error of the mean of four (without cells) or five (with cells) independent experiments.

Chapter IV

Conclusions

Conclusions

The work presented in this dissertation provides an increased knowledge of the chemical profile and the biological potential of *E. plantagineum* bee pollen.

The main conclusions are listed below.

Hydromethanol extract:

- *S*ignificant amounts of phenolic compounds were found.
- Eight organic acids and five fatty acids were determined for the first time.
- *In vitro* assays revealed the ability to scavenge $^{\bullet}NO$ and $O_2^{\bullet-}$, although for the latter a pro-oxidant behaviour was observed for the higher concentrations tested.
- The extract reduces $^{\bullet}NO$ and prostaglandins release in LPS-stimulated macrophages, but, in what concerns to prostaglandins, this is true only if the extract concentrations are maintained below certain levels.
- The levels of prostaglandins metabolites derived from Cyt P450 enzymes, like 20-hydroxy-PGE2 and 6-keto-PGF1α, are reduced by high extract concentrations. However, under those conditions several isoprostanes are increased.
- The extract is effective in inhibiting basophils degranulation under an allergic stimulus, as ascertained by the levels of β-hexosaminidase released. Nevertheless, some deleterious effects on insulted cells were noticed at the highest tested concentrations.
- Reduced antibacterial capacity was observed and just against *S. aureus*, *S. epidermidis* and *M. luteus*.

Acidified methanol extract and fractions:

- Seven flavonols and five anthocyanins were determined.
- The phenolic extracts were not efficient at protecting Caco-2 cells from the oxidative stress induced by *t*-BHP, although the removal of anthocyanins ceased the pro-oxidant effects and imparted a small protection.

- The antioxidant potential of the compounds may be abrogated by the fact that in the presence of iron *t*-BHP generates reactive species, which can be more deleterious than the ones generated by its biotransformation.

- The pro-oxidant effects seen with the extracts containing anthocyanins may also be ascribed to their ease in being themselves converted into radical species.

- The protective effects seem to be related with the levels of total glutathione.

Overall, the results obtained demonstrate that *E. plantagineum* bee pollen bioactive compounds can impart beneficial health effects, by reducing the levels of oxidative stress and inflammatory mediators involved in the genesis of many diseases, and also by preventing allergy and ameliorating allergy symptoms.

Chapter V

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