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The neuroprotective potential of medicinal species: From flavonoid-rich extracts to nanophytosomes with multimodal effects

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The neuroprotective potential of medicinal species: From flavonoid-rich extracts to nanophytosomes with multimodal effects

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***“(...) E uma infinita tristeza,
uma funda turbção
entra em mim, fica em mim presa.
Cai neve na Natureza
- e cai no meu coração.”***

Augusto Gil
Balada da Neve, in Luar de Janeiro (1909)

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ABSTRACT

ABSTRACT

Medicinal plants are a valuable source of bioactive compounds, including flavonoids. Epidemiological studies demonstrate an inverse association between flavonoid intake and risk of occurrence of neurodegenerative disorder, such as Alzheimer's and Parkinson's diseases. A large body of evidence demonstrates that flavonoids interact with multiple targets and pathways in the Central Nervous System resulting in a broad neuroprotective effect mediated by complementary processes, and that flavonoid-based therapies may input positive outcomes in the prevention and early management of neurodegenerative disorders. However, the low bioavailability of flavonoids is frequently pointed as the main pitfall refraining their therapeutical application. The development of lipid-based nanocarriers is a valuable strategy to enhance the brain bioavailability of flavonoids and their neuroprotective effects.

Trichilia catigua A.Juss and *Turnera diffusa* Willd. ex Schult. were the two medicinal species selected to this study for their traditional use as neurostimulants, which is frequently associated with their phenolic composition. In *T. catigua* stem bark crude aqueous extract were identified twenty-six phenolic compounds (551.02 ± 37.61 mg/g of lyophilized extract). The majority were flavonoids, namely flavan-3-ols, their oligomers and phenylpropanoid derivatives, the last group representing ~81% of the total quantified mass. In *T. diffusa* leave crude aqueous extract were identified thirty-seven phenolic compounds (18.20 ± 2.12 mg/g of lyophilized extract), all flavonoids, from which twenty-six were described for the first time. Luteolin derivatives were the dominant flavonoids, corresponding to ~68% of the total quantified mass.

The *in vitro* neuroprotective effects of the crude extracts were assessed by their capacity to interact with common targets and pathways underpinning neurodegenerative disorders. *T. catigua* extract excelled in terms of antiradical effects (scavenging of DPPH $\cdot$, $\cdot$ NO and O $_2^{\cdot-}$ radicals, and suppression of lipid peroxidation), enzyme inhibition (xanthine oxidase, monoaminoxidase-A, tyrosinase, cholinesterases and 5-lipoxygenase) and antiglycation activity. In the assays conducted with SH-SY5Y cells, the pre-treatment with both extracts protected cells against 6-hydroxydopamine/ascorbic acid (6-OHDA/AA)-induced toxicity, although *T. catigua* extract was active at lower concentrations. In contrast, the pre-treatment with the extracts failed to protect SH-SY5Y cells against glutamate-induced toxicity. In BV-2 microglia cells, only *T. diffusa* extract mitigated interferon- γ pro-inflammatory insult, as observed by the reduction of NO production.

A new chloroform-free protocol was implemented to extract egg yolk phospholipids and was obtained a phospholipid-rich fraction of high purity degree (phospholipid content of $208.65 \pm 26.46 \mu\text{mol/g}$ fresh egg yolk, accounting for *ca.* 96% of the extract's dry weight). It also contained cholesterol ($0.069\text{--}0.082$ cholesterol/phospholipid molar ratio), and vestigial amounts of lutein and zeaxanthin (89.24 ± 9.76 and $14.9 \pm 2.16 \text{ ng/g}$ of fresh egg yolk, respectively). Saturated fatty acids dominated in the extracted phospholipids (50% of egg's total yolk phospholipids), the levels of monounsaturated ranged from 20 to 25%, and of polyunsaturated up to 35%.

Rutin-liposomes were prepared with the phospholipid-rich fraction. They presented mean diameter $< 140 \text{ nm}$, negative surface charge of *ca.* -13 mV , and entrapment efficiency up to 87%. The interactions of rutin with the phospholipid membrane suggested that these lipid vesicles were suitable to entrap other flavonoids, or flavonoid-rich extracts. The pre-treatment of SH-SY5Y cells with rutin-liposomes attenuated glutamate-induced cytotoxicity, in part by reducing the formation of intracellular reactive species.

T. catigua and *T. diffusa* flavonoid-rich extracts were obtained through solid-phase extractions of the initial crude extracts. They were combined with the phospholipid-rich fraction to produce nanophytosomes. The distinct phenolic composition of each extract deeply conditioned the physicochemical characteristics of the nanostructures, although all presented suitable features to possibly enhance the brain bioavailability of the extracts. The study of the stability profile showed that *T. diffusa* nanophytosomes are more stable than *T. catigua* nanophytosomes. Furthermore, the long-term stability of the formulations can be improved if they are subjected to dialysis with mannitol followed by lyophilization. The pre-treatment of SH-SY5Y cells with the nanophytosomes did not protect cells from 6-OHDA/AA-induced toxicity but, in contrast, protected them from glutamate-induced toxicity. On the other hand, only the pre-treatment of BV-2 cells with *T. diffusa* nanophytosomes protected them from interferon- γ insult in a concentration-dependent effect, as observed by the reduction of NO levels.

Overall, these results highlight the value of plants as sources of bioactive flavonoids with neuroprotective potential mediated by a multitarget effect. Furthermore, these results also support the development of lipid-based nanocarriers for flavonoid-rich extracts, and their possible application as neuroprotective agents in the management of neurodegenerative disorders.

Keywords: *Trichilia catigua*; *Turnera diffusa*; flavonoids; liposomes; phytosomes; neurodegenerative disorders; neuroprotection.

RESUMO

RESUMO

As plantas medicinais são uma fonte valiosa de compostos bioativos, incluindo os flavonoides. Estudos epidemiológicos demonstram uma associação inversa entre a ingestão de flavonoides e o risco de ocorrência de doenças neurodegenerativas, como as doenças de Alzheimer e Parkinson. Os flavonoides interagem com vários alvos e vias no Sistema Nervoso Central, resultando em um amplo efeito neuroprotetor mediado por processos complementares, e há evidência que terapias baseadas em flavonoides podem impedir a progressão destas doenças. A baixa biodisponibilidade dos flavonoides é frequentemente apontada como o principal entrave à sua aplicação terapêutica. No entanto, o desenvolvimento de nano transportadores à base de lipídios é uma possível estratégia para aumentar a sua biodisponibilidade, bem como os seus efeitos neuroprotetores.

As espécies *Trichilia catigua* A.Juss e *Turnera diffusa* Willd. ex Schult. foram selecionadas para este estudo pelo seu uso tradicional como neuroestimulantes, o qual é frequentemente associado à composição fenólica. No extrato aquoso bruto de cascas de *T. catigua* foram identificados vinte e seis compostos fenólicos ($551,02 \pm 37,61$ mg/g de extrato liofilizado), a maioria flavan-3-óis, seus oligômeros e derivados fenilpropanoides, os últimos representando ~ 81% da massa total quantificada. No extrato aquoso bruto de folhas de *T. diffusa* foram identificados trinta e sete flavonoides ($18,20 \pm 2,12$ mg/g de extrato liofilizado), a maioria derivados da luteolina que correspondem a ~ 68% da massa total quantificada.

Os efeitos neuroprotetores dos extratos foram avaliados *in vitro* em alvos e vias comuns às doenças neurodegenerativas. O extrato de *T. catigua* destacou-se em relação aos efeitos antirradicais (supressão dos radicais DPPH $\cdot$, $\cdot$ NO e $O_2^{\cdot-}$, e da peroxidação lipídica), inibição enzimática (xantina oxidase, monoamino oxidase-A, tirosinase, colinesterases e 5-lipoxigenase) e atividade antiglicante. Nos ensaios conduzidos com as células SH-SY5Y, o pré-tratamento com ambos os extratos protegeu as células contra a toxicidade induzida pela 6-hidroxidopamina/ácido ascórbico (6-OHDA/AA), embora o extrato de *T. catigua* seja mais ativo em concentrações mais baixas. Em contraste, o pré-tratamento com os extratos não protegeu estas células contra a toxicidade induzida pelo glutamato. Em células da microglia BV-2, apenas o extrato de *T. diffusa* mitigou a agressão pró-inflamatória do interferon- γ , conforme observado pela redução da produção de óxido nítrico.

Foi desenvolvido um novo protocolo, sem clorofórmio, para a extração de fosfolípidos da gema de ovo, sendo obtida uma fração rica em fosfolípidos com alto grau de pureza (cerca de 96% do peso seco do extrato). Era constituída também por colesterol (razão molar de

0,069-0,082 colesterol/fosfolípido) e quantidades vestigiais de luteína e zeaxantina ($89,24 \pm 9,76$ e $14,9 \pm 2,16$ ng/g de gema de ovo fresca, respectivamente). Os ácidos gordos saturados dominaram entre os fosfolípidos extraídos (50% do total de fosfolípidos), os níveis dos monoinsaturados variaram entre 20 e 25% e dos polinsaturados corresponderam até 35%.

Os nanolipossomas contendo rutina foram preparados com a fração rica em fosfolípidos, e apresentaram um diâmetro médio <140 nm, carga superficial negativa de ca. -13 mV e uma eficiência de encapsulação até 87%. As interações entre a rutina e membrana fosfolipídica sugeriram que as vesículas lipídicas são adequadas para encapsular flavonoides, ou extratos ricos em flavonoides. O pré-tratamento de células SH-SY5Y com os nanolipossomas com rutina atenuou a citotoxicidade induzida por glutamato, em parte através redução da formação de espécies reativas intracelulares.

Extratos ricos em flavonoides foram obtidos através de extrações em fase sólida partindo dos extratos brutos iniciais de *T. catigua* e *T. diffusa*. Estes foram combinados com a fração rica em fosfolípidos para a produção de nanofitossomas. A composição fenólica de cada extrato condicionou consideravelmente as propriedades físico-químicas das nano estruturas, embora todas apresentassem características adequadas para o possível aumento da biodisponibilidade dos extratos. O estudo do perfil de estabilidade mostrou que os nanofitossomas de *T. diffusa* são mais estáveis do que os de *T. catigua*, e que a estabilidade a longo prazo pode ser melhorada se estes forem submetidos a diálise com manitol, seguida de liofilização. O pré-tratamento das células SH-SY5Y com os nanofitossomas não protegeu as células da toxicidade induzida pela 6-OHDA/AA, mas, pelo contrário, protegeu-as da toxicidade induzida pelo glutamato. Apenas o pré-tratamento das células BV-2 com os nanofitossomas de *T. diffusa* protegeu as células da agressão com interferon- γ , num efeito dependente da concentração, conforme observado pela redução dos níveis de óxido nítrico.

No geral, estes resultados reforçam o potencial neuroprotetor dos flavonoides. Além disso, também suportam o desenvolvimento de nano transportadores à base de lípidos para extratos ricos em flavonoides e sua possível aplicação como agentes neuroprotetores no tratamento de doenças neurodegenerativas.

Palavras-chave: *Trichilia catigua*; *Turnera diffusa*; flavonoides; lipossomas; fitossomas; doenças neurodegenerativas; neuroproteção.

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LIST OF ABBREVIATIONS

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5-HT	Serotonin (5-hydroxytryptamine)
6-OHDA/AA	6-Hydroxydopamine/ascorbic acid
16-AP	16-(9-Anthroyloxy)palmitic acid
A β	Amyloid- β
AAE	Ascorbic acid equivalents
ABC	ATP-binding cassette
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AGEs	Advanced glycation end-products
Akt/PKB	Akt/protein kinase B
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APP	Amyloid precursor protein
AS	Aureusidin synthase
ATCI	Acetylthiocholine iodide
BBB	Blood-brain barrier
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
BTCI	<i>S</i> -butyrylthiocholine iodide
CaMK	Calcium calmodulin kinase
cAMP	Cyclic adenosine monophosphate
CCD	Charge-coupled device
CCL2	C-C motif chemokine ligand 2
CNS	Central nervous system
CoA	Coenzyme A
COMT	Catechol- <i>O</i> -methyltransferase
COX	Cicloxygenase

CREB	cAMP-response element-binding protein
CYP450	Cytochrome P450 enzyme superfamily
DA	Dopamine
DAD	Diode array detector
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DFR	Dihydroflavonol 4-reductase
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DMID	7,2'-Di-hydroxy-4'-methoxy-isoflavonol dehydratase
DPPE	1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine
DPPH·	1,1-Diphenyl-2-picrylhydrazyl free radical
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EAAT	Excitatory amino acid transporters
EDTA	Ethylenedinitrilotetraacetic acid
EE	Entrapment efficiency
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
FAME	Fatty acid methyl esters
FBS	Fetal bovine serum
FID	Flame ionization detector
F ₃ H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
FS _{1/2}	Flavone synthase 1/2
GABA	gamma-Hydroxybutyric acid
GC	Gas chromatography
GLUT1	Glucose transporter 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione

HBSS	Hank's Balanced Salt Solution
HD	Huntington's disease
HEPES	4-(2-Hydroxyethyl)piper-azine-1-ethanesulfonic acid
HPLC	High performance liquid chromatography
IFR	Isoflavone reductase
IFS	Isoflavone synthase
IFN- γ	Interferon gamma
iGluR	Ionotropic glutamate receptor
IL	Interleukin
IS	Internal standard
I ² H	Isoflavone 2'-hydroxylase
JNK/SAPK	Stress-activated protein kinases/Jun amino-terminal kinases
LAR	Leucoanthocyanidin reductase
LC	Liquid chromatography
L-DOPA	3,4-Dihydroxy-L-phenylalanine
LDOX	Leucoanthocyanidin dioxygenase
LogP	Partition coefficient
LOX	Lipoxygenase
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
mGluR	Metabotropic glutamate receptors
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NA	Noradrenaline
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium chloride

NDDS	Novel drug delivery systems
NFκB	Nuclear factor κB
NIR	Near infrared
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NOX	NADPH oxidase
NMDA	N-methyl-D-aspartate
OMT	O-Methyltransferase
P _{app}	Apparent permeability coefficient
PC	Phosphatidylcholine
PD	Parkinson's disease
P-gp	P-glycoprotein
PI-3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMS	Phenazine methosulfate
PUFA	Polyunsaturated fatty acids
QTOF	Quadrupole time-of-flight
RAGE	Receptor for advanced glycation end-product
RhamT	Rhamnosyl transferase
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SOD	Superoxide dismutase
SGLT	Sodium-dependent glucose transporter
SLC	Solute carriers
SNP	Sodium nitroprusside dehydrate
SPE	Solid phase extraction
STAT	Signal transducer and activator of transcription

TAC	Total antioxidant capacity
T&CM	Traditional and Complementary Medicine
TEM	Transmission electron microscopy
TGF- β	Transforming growth factor- β
TLC	Thin-layer chromatography
TLR	Toll-like receptors
TNF- α	Tumour necrosis factor- α
TrkB	Tropomyosin receptor kinase B
UDP	Uridine-5'-diphosphate
UGT	UDP-glucuronosyltransferase
UFGT	UDP flavonoid glucosyl transferase
UHPLC	Ultra-high performance liquid chromatography
VR	Vestitone reductase
XO	Xanthine oxidase
WHO	World Health Organization

THESIS OUTLINE

THESIS OUTLINE

This PhD thesis is composed by five main sections:

CHAPTER I – Introduction and Objectives

This chapter provides the state-of-the-art of the main topics explored in the workplan of the thesis, including information required to understand and interpretate the experimental findings. The main objectives of this thesis are listed at the end of this chapter.

CHAPTER II – Experimental Section

This chapter details all the information about the used materials and plant material, and the protocols performed to collect experimental data.

CHAPTER III – Results and Discussion

This chapter comprehends the exposition of all the experimental data, and the discussion of results in light of the current knowledge.

CHAPTER IV – Conclusions

The main conclusions drawn from this work are summarized in this chapter.

CHAPTER V – References

This chapter contains all the bibliographic references cited in the writing of this thesis.

CHAPTER I

Introduction

Objectives

1. Introduction

1.1. Medicinal plants: resourceful source of drugs

The search for drugs in Nature is a primordial practice. Since early periods of human civilization, the healing properties of certain medicinal plants have been evidenced and this knowledge was continuously systematized, upgraded and conveyed to the succeeding generations until present days (1). Some examples of these ancient, but sophisticated, systems are the Ayurvedic Medicine and the Chinese Traditional Medicine. This last one, in its magnitude, includes 11 146 species of medicinal plants belonging to 383 families and 2309 genera (2).

The use and study of medicinal plants have been constantly, and deeply, influencing the Western medicine. The definition of Pharmacognosy (merge of two Greek words *pharmakon* (drug) and *gnosis* (knowledge), i.e., the knowledge of drugs) as a discipline in 1815 by Seidler was part of the progress, and this was by far the most important pharmaceutical discipline and the basis for many others (3). Of the approximately 270 000 terrestrial plants taxonomically classified, at least 10 000 higher plants have medical relevance (4), and plant-derived natural products found in the diverse pharmacotherapeutic sub-classes represent no less than 25% of all the drugs in clinical use in the world (5).

The use of plants, plant extracts or plant-derived chemicals is not an outdated routine, nor even an exclusive stronghold for the pharmaceutical industries. In the past decades, the revivalism of phytotherapy launched the market of herbal products, which are consumed worldwide and flourishing in the developed countries. In 2017, this global market is valued around 1 trillion dollars and is growing annually by 8 to 10% (6). The public interest and acceptance of natural therapies, including phytotherapy, has become mainstream across the European countries and herbal products are available not only in pharmacies under supervision, but also in herbal stores, food stores and supermarkets (7).

Even today, Traditional and Complementary Medicine (T&CM), in its majority based on the use of medicinal plants, is still the first-line treatment and often the only one accessible for millions in developing countries. In an effort to achieve the world recognition and validation of T&CM, the World Health Organization (WHO) continuously issues reports and traces new strategies in this matter. WHO recognizes T&CM as a vital part of the global health care system: the release of the WHO Traditional Medicine Strategy 2014–2023 (an evaluation planned halfway through its 10-year lifespan) was projected to raise awareness,

provide recommendations to the governments to harness the potential contribution of T&CM, and, also, to urge for concise regulation and substantiating scientific research (8).

It is in this framework, the duality and the co-existence of empiric traditional medicine and scientific research, that an opportunity arises for a converging path with the purpose of exploration of the vast potential of medicinal plants. Ethnopharmacological investigations have long proved its significance in plant-based drug discovery by taking advantage of the interdisciplinary scientific study in combination with field observations and descriptions of the utilization of folk remedies (9). Back in 2001, in a survey to assess the value of plants used in traditional medicine for drug discovery was reported that from the 122 plant-derived drugs currently used in pharmacotherapy, approximately 80% have had an ethnomedical use identical or related to the current use of the active elements of the plant (10).

In drug discovery from medicinal plants, the ethnopharmacological investigation is the classical approach but a variety of other knowledge-based strategies can be applied to increase the probability for the identification of relevant bioactive compounds: the chemosystematic approach, which consists in selecting the test samples based on chemotaxonomy and phylogeny by taking into account that plant species from the same genera or families are known to produce compounds associated with a certain bioactivity; the principal of the ecological approach is the selection based on the interactions between organisms and their environment, considering that plant secondary metabolites possess ecological functions that may also derive into potential therapeutic application; the computational approach that consists in selecting test samples relying on *in silico* bioactivity predictions for constituents of certain plant species (11). The gradual advances in the analytical field also led to the refinement and development of new techniques useful in drug discovery. Some examples are the new extraction techniques, the application of chromatographic and spectroscopic methods and the hyphenation of these techniques, or data processing using multivariate analysis software that opened metabolomics and *in silico* analysis (12). The process of drug discovery from herbal medicines is often considered a long and endeavour experience. However, the success rate of the synthetic route may be 1/10 000, while the success rate of searching for new therapeutic moieties based on medical plants used in traditional medicinal systems can be as high as 1/4, or more (13).

Overall, the identification and isolation of new compounds from medicinal species, as well as and the study of their pharmacological effects are an enduring and overwhelming opportunity to expand the existing drug arsenal (14).

1.1.1. Plant bioactive compounds

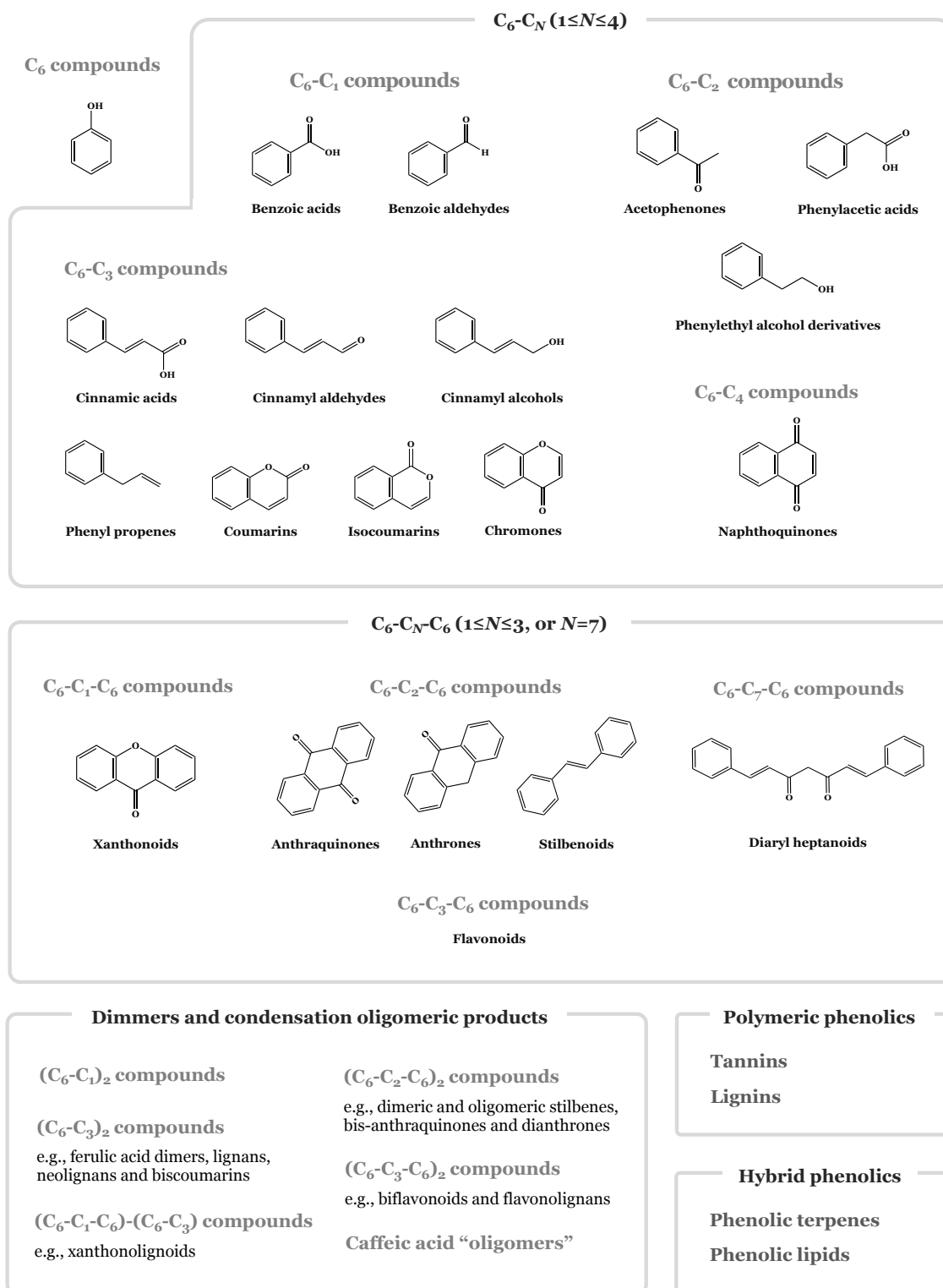
In general, plant metabolites are classified according to their role in the metabolic processes as primary or secondary metabolites. Primary metabolites are those involved in fundamental plant physiological processes and are directly required for plant growth, while secondary metabolites mediate plant–host/environment interactions (15). In addition to hormones, which regulate organismal processes and metabolism, these three groups are considered as the functional trichotomy of plant metabolism (16). However, the notion of the exact boundaries between the different classes has been updated to a more integrated blurred vision of the multifunctionality of secondary metabolites.

Secondary plant metabolites are the key-drivers behind the pharmacological action of medicinal plants. They often occur exclusively in certain species because they are biosynthesized in specific cells/organs and constitute an extremely large group, including compounds from diversified classes, such as alkaloids, terpenes, saponins, lipids, carbohydrates, and phenolic compounds.

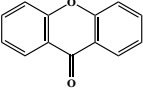
Among the most studied secondary metabolites are phenolic compounds (17). Within these, distinct structures are found, comprising simple phenolics, bearing one hydroxylated aromatic ring, up to large polyphenolic structures and polymers (**Figure 1**). In fact, due to their complex structural variety, the definition of phenolic compound has been redefined to a more comprehensive description of plant secondary metabolites biosynthesized exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathways, featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression, which fulfil a very broad range of physiological roles in plants (18). These compounds can also be found as heterosides, when the aglycone is linked to sugar residues, such as glucose or rhamnose, either by their hydroxyl groups or by carbon atoms of the main skeleton. Plant phenolics exhibit a remarkable diverse range of bio-physicochemical properties that makes them rather unique and intriguing natural products. In plants, they are implicated in many diverse functional roles. As example, they have physiologic relevance as a way of channelling and storing carbon compounds accumulated from photosynthesis during periods when nitrogen is scarce, they establish colour and contribute to certain flavours, take part in resistance mechanisms against microbial pathogens and herbivores, and protect from solar damaging radiation (19).

These secondary metabolites are ubiquitous in all plants. Therefore, despite their functional roles in the plant they are an integral part of the human diet. The impact of their consumption on human health has emerged as field of interest for the last decades, and

current literature suggests that the long-term consumption of diets rich in phenolic compounds may have preventive or therapeutic effects in cardiovascular diseases, type 2 diabetes, cancer, or even neurodegenerative diseases (20,21).

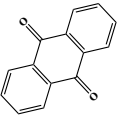


$C_6-C_1-C_6$ compounds

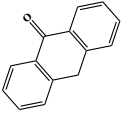


Xanthonoids

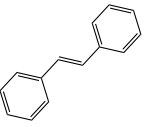
$C_6-C_2-C_6$ compounds



Anthraquinones

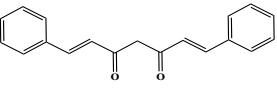


Anthrones



Stilbenoids

$C_6-C_7-C_6$ compounds



Diaryl heptanoids

Dimmers and condensation oligomeric products

$(C_6-C_1)_2$ compounds

$(C_6-C_3)_2$ compounds
e.g., ferulic acid dimers, lignans, neolignans and biscoumarins

$(C_6-C_1-C_6)-(C_6-C_3)$ compounds
e.g., xanthonolignoids

$(C_6-C_2-C_6)_2$ compounds
e.g., dimeric and oligomeric stilbenes, bis-anthraquinones and dianthrones

$(C_6-C_3-C_6)_2$ compounds
e.g., biflavonoids and flavonolignans

Caffeic acid “oligomers”

Polymeric phenolics

Tannins

Lignins

Hybrid phenolics

Phenolic terpenes

Phenolic lipids

Figure 1. Classification of phenolic compounds in plants (adapted from (16)).

1.1.2. A special focus on flavonoids

Flavonoids are a sub-class of phenolic compounds widely distributed in the plant kingdom, and one of the most characteristic compounds in higher plants. This group comprises a large number of compounds, which is estimated to be over 9 000 different structures, and the list continues expanding (22). Flavonoids share a basic diphenyl propane carbon skeleton ($C_6-C_3-C_6$) and are classified based on the different patterns of oxidation of their common structure as chalcones, flavanones, flavones, isoflavones, aurones, flavonols, anthocyanidins, flavan-3-ols, and flavan-3,4-diols (**Figure 2**). They are synthesized in the cytosol and transported into the vacuole for storage, or to other destinations.

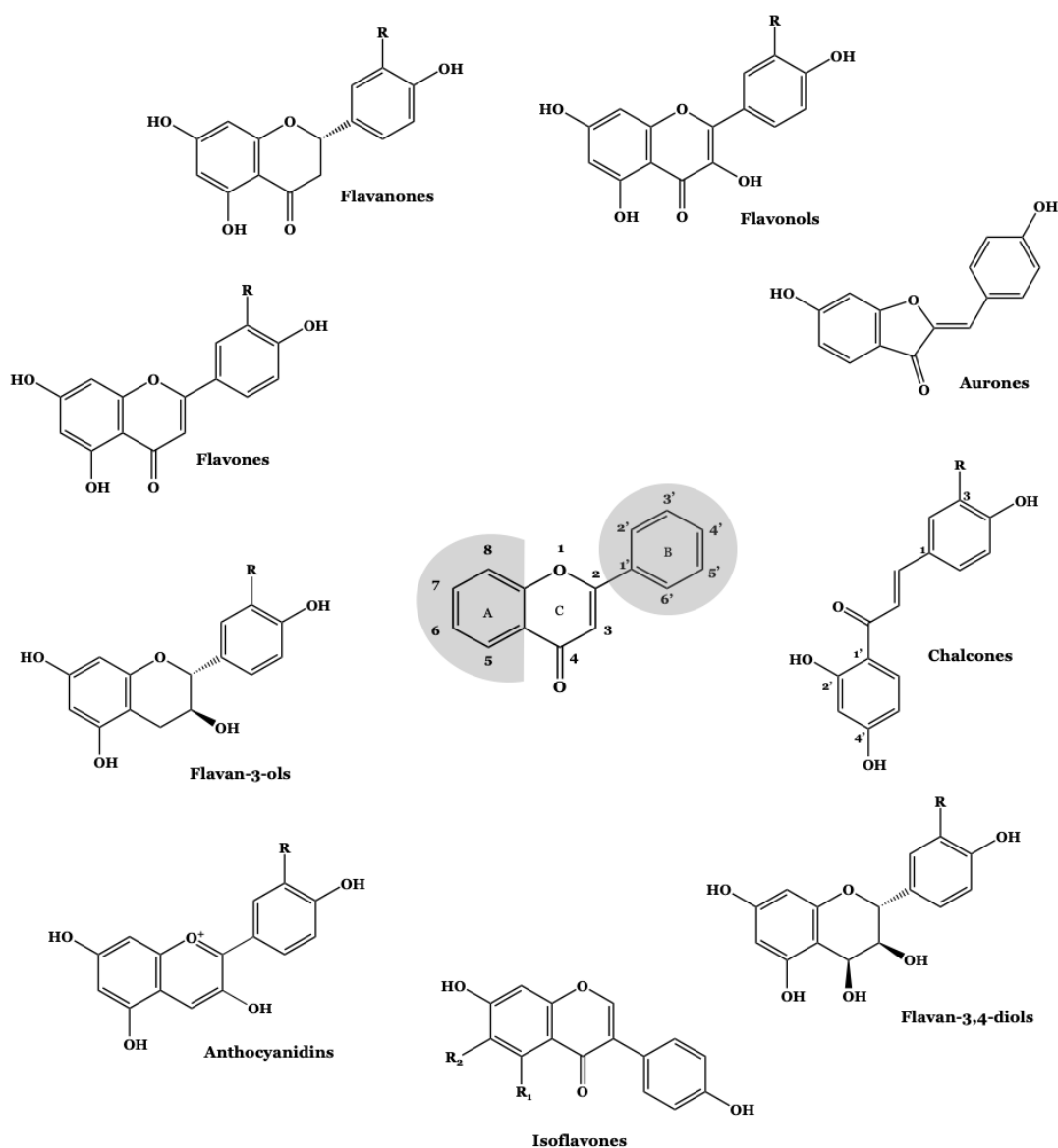


Figure 2. General backbone of flavonoids and main sub-classes.

Flavonoids play a wide variety of significant roles in plants, which span from physiologic effects to mediation of plant-environment interactions. For instance, they are engaged in plant reproduction and fertility, UV photoprotection, they act as signalling molecules in response to symbiont or pathogenic microorganisms (e.g., as phytoalexins), and to abiotic stress (e.g., drought stress, nitrogen, and temperature), they are mediators in plant-plant communication and in plant interactions with insects, either as attractants (e.g., attraction of pollinators *via* flower colours) or as deterrents to herbivores (23–25).

1.1.2.1. Biosynthesis and classification

The distribution in the plant kingdom of flavonoids and their biosynthetic genes suggests that flavonoid biosynthetic pathways evolved through a series of steps (26).

Flavonoids are derived from two metabolic pathways: shikimate and acetate. In the general phenylpropanoid pathway, the deamination of phenylalanine is catalysed by phenylalanine ammonia-lyase to form cinnamic acid (phenylpropanoid skeleton) (**Figure 3**). Cinnamic acid 4-hydroxylase then catalyses the introduction of a hydroxyl group at the *para* position of the phenyl ring of cinnamic acid to produce *p*-coumaric acid (**Figure 3**). In a process catalysed by *p*-coumaroyl:CoA ligase, the carboxyl group of *p*-coumaric acid is activated by formation of a thioester bond with Coenzyme A (CoA) to form *p*-coumaroyl-CoA (**Figure 3**), which is one of the precursors of flavonoid biosynthesis (27).

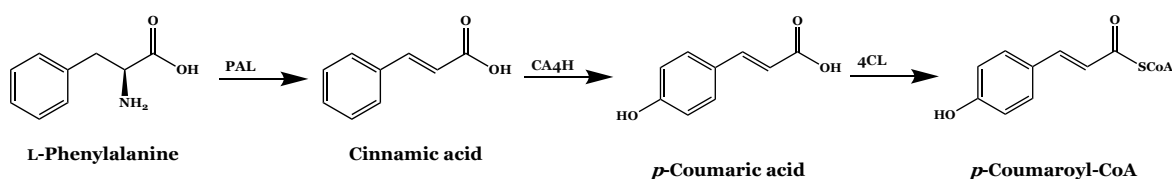


Figure 3. The general phenylpropanoid pathway leading from phenylalanine to *p*-coumaroyl-Coenzyme A (CoA). PAL, phenylalanine ammonia-lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, *p*-coumaroyl:CoA ligase.

The other precursor is malonyl-CoA provided by the acetate pathway. This essential substrate for flavonoid biosynthesis is produced in the cytosol through carboxylation of acetyl-CoA by acetyl-CoA carboxylase (28).

The flavonoid biosynthetic grid is depicted in **Figure 4**. Although the central pathway for flavonoid biosynthesis is conserved in plants, three main group of enzymes mediate the

structural modifications of the basic flavonoid skeleton, leading to the distinct sub-classes: NADH-dependent reductases are dihydroflavonol 4-reductase (DFR), leucoanthocyanidin reductase (LAR), isoflavone reductase (IFR) and vestitone reductase (VR); cytochrome P450 hydroxylases are flavonoid 3'-hydroxylase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), isoflavone synthase (IFS), isoflavone 2'-hydroxylase (I2'H) and flavone synthase (FS1/FS2); Fe²⁺/2-oxoglutarate-dependent dioxygenases are flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), flavone synthase (FS1) and leucoanthocyanidin dioxygenase (LDOX) (23).

The first step in flavonoid biosynthesis is the condensation of *p*-coumaroyl-CoA (contributes with nine carbons, forming the B- and C-rings) with three malonyl-CoA units (six carbons of the A-ring) in a polyketide chain elongation reaction followed by cyclization of the A-ring, catalysed by chalcone synthetase and resulting in a chalcone (29). Subsequently, the enzyme chalcone isomerase (CHI) catalyses the stereospecific intramolecular cyclization of the chalcone to the respective flavanone (2S)-naringenin, which is the common substrate for the various flavonoid branch pathways.

Isoflavones are derived from flavones in two steps. Initially, there is a migration of 1,2-aryl group along with C-2 hydroxylation to form 2-hydroxy-isoflavone, in a reaction catalysed by IFS. Then the introduction of a double bound between C-2 and C-3 occurs, being catalysed by a dehydratase (30). The enzymes isoflavone 2'-hydroxylase (I2'H), IFR, VR and 7,2'-di-hydroxy-4'-methoxy-isoflavonol dehydratase (DMID) are involved in the biosynthesis of isoflavonoids from isoflavones (31,32).

Flavones are biosynthesized from flavanones. The introduction of a double bond between C-2 and C-3 is either mediated by FS1 that abstracts two electrons from C-2 and C-3, or by FS2 that catalyses the formation of a hypothetic 2-hydroxyflavanone that is further dehydrated to flavone (33).

The oxidation of flavanones to dihydroflavonols (3-OH-flavanones) is catalysed by F3H. Then the enzyme FLS mediates the unsaturation of (2R,3R)-*trans*-dihydroflavonols to form flavonols (34). The hydroxylation of B-ring is determined by the activity of F3'H (C-3' hydroxylation) and F3'5'H (C-3' and C-5' hydroxylation) (33).

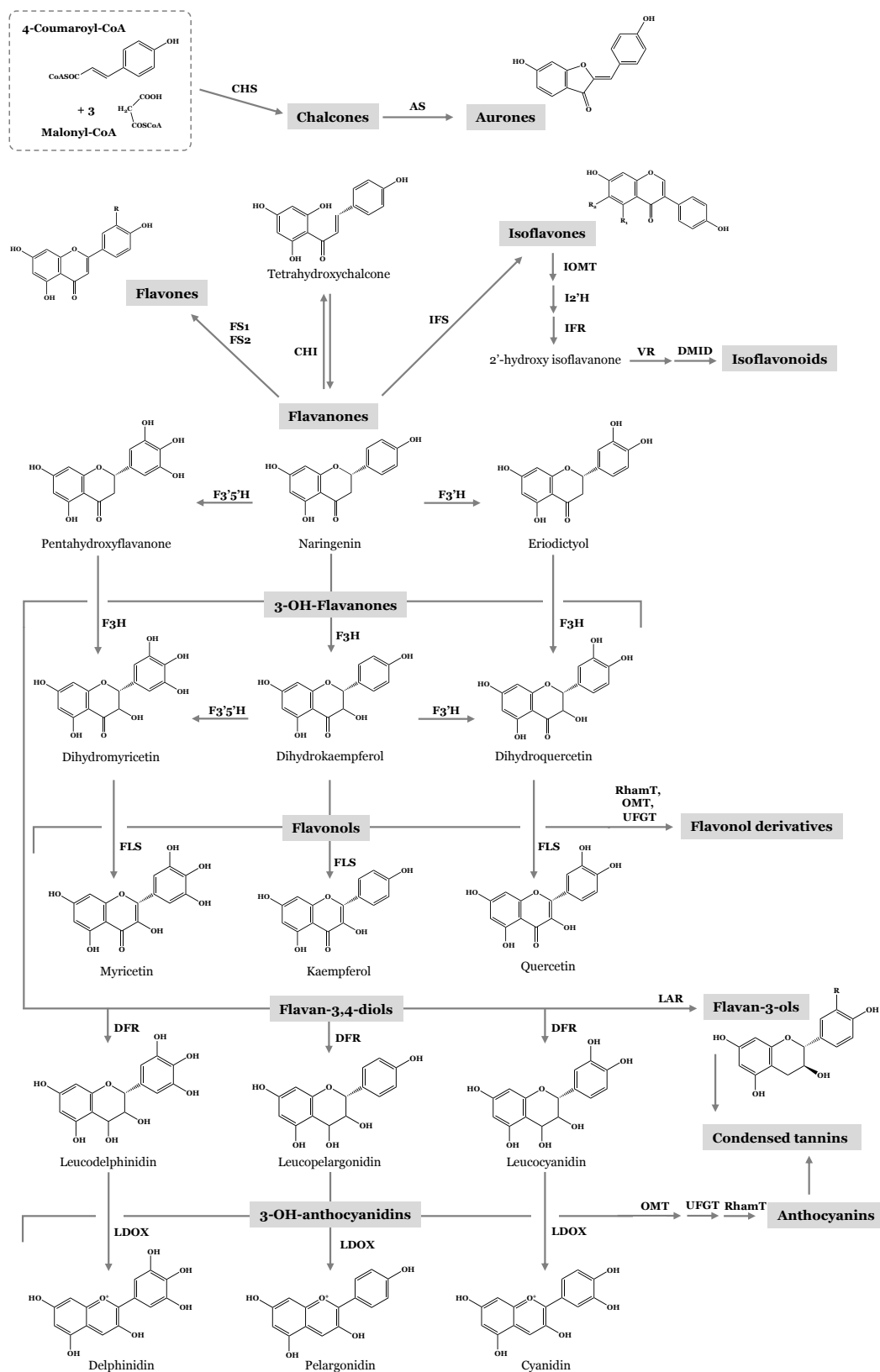


Figure 4. Scheme of the biosynthesis of the main flavonoid sub-classes (adapted from (35)). AS, aureusidin synthase; DFR, dihydroflavonol 4-reductase; DMID, 7,2'-dihydroxy,4'-methoxyisoflavanol dehydratase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3,5' hydroxylase; FLS, flavonol synthase; FS1/FS2, flavone synthase; I2'H, isoflavone 2'-hydroxylase, IFR, isoflavone reductase; IFS, isoflavone synthase; IOMT, isoflavone O-methyltransferase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; OMT, O-methyltransferase; RhamT, rhamnosyl transferase; UFGT, UDP flavonoid glucosyl transferase; VR, vestitone reductase.

The enzyme DFR catalyses the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanidins). These are also substrates for the biosynthesis of anthocyanins *via* 3-OH-anthocyanins mediated by LDOX, and the 3-O-glycosyltransferases (UDP flavonoid glucosyl transferase (UGT) and rhamnosyl transferase (RhamT)). The enzyme LAR catalyses the biosynthesis of flavan-3-ols, which are monomers in the formation of condensed tannins (proanthocyanidins), from flavan-3,4-diols (33).

The enzyme aureusidin synthase (AS) is a polyphenol oxidase homolog that catalyses the hydroxylation and oxidative cyclization of chalcones to form aurones (36).

Condensed tannins, also known as proanthocyanidins, are a distinct group phenolic compounds, consisting of oligomers ($n = 2-7$) or polymers ($n = 8-24$, or more) of flavan-3-ol units. Details of their synthetic mechanism remain unclear, for example, how do flavan-3-ol units polymerize to produce proanthocyanidins and whether this polymerization is enzyme-mediated, or not (37). Proanthocyanidins are classified according to the differences in hydroxylation patterns into several subgroups, for instance procyanidins designate oligomers and polymers with 3,3',4',5,7-hydroxyl pattern ((+)-catechin and/or (-)-epicatechin units). These compounds are linked between the C4 position of the upper unit and the C8 or C6 position of the lower unit, and the type of interflavan linkage, α or β : in procyanidins the interflavan linkage between C4 and C8 positions are stereochemically predominant, C4→C8 and C4→C6 linkages usually being present in a ratio of 3:1; the proanthocyanidins composed of flavan-3-ol units linked mainly through C4→C8 and/or C4→C6 are categorized as B-type; the flavan-3-ol units can also be doubly linked by an additional ether bond between C2 position of the upper unit and the oxygen at C7 or C5 position of the lower unit; the oligomers or polymers which contain both C2 β →O→7 ether-type and C4→C8 or C4→C6, as well as those which contain both C2 β →O→5 ether-type and C4→C6 are categorized as A-type (37).

In the final steps of flavonoid biosynthesis, their structures may be modified by the addition of sugars (glycosylation), hydroxyl groups (hydroxylation), methyl groups (methylation) and acyl moieties (acylation). The most common modification is hydroxylation (occurring in 78.6 % of flavonoids), being followed by glycosylation (56.2%), methylation (24.8%) and phenylacylation (4.8%) (38). These structural modifications create molecules with higher stability and modify their bioavailability and their bioactivities, as well. In hydroxylation reactions, hydroxyl groups are introduced *via* substitution of functional groups or hydrogen atoms and are usually catalysed by either cytochrome P450-family enzymes or by Fe²⁺/2-oxoglutarate-dependent dioxygenases (38). Other common structural modification is the methylation of hydroxyl groups.

Methyltransferases attach methyl moieties to the aglycone to form methoxides *via* oxygen or carbon atom, originating *O*-methylated or *C*-methylated compounds, respectively (39). The glycosylation of the flavonoid core structures also occurs at various stages of their biosynthesis *via* uridine diphosphate (UDP) glycosyltransferases. These enzymes transfer UDP-activated sugar donor molecules (e.g., UDP-glucose, UDP-glucuronic acid, UDP-rhamnose) onto an acceptor, such as hydroxyl groups (27). This conjugation may be independent (monoglucosides), in parallel or in chain (di-, tri-glucosides, etc). Flavonoid heterosides are often found acylated with aliphatic and aromatic acids (mainly acetic, malonic, *p*-coumaric, and ferulic acids), since the hydroxyl groups of their sugar moieties can undergo acylation by acyltransferases (40).

1.1.2.2. Extraction and analysis

Flavonoids can be extracted from the most diversified natural sources, in particular, from plant material such as flowers, leaves, barks, stems, fruit, and peels, or even by using the whole plant. In addition, it may be projected the extraction of a specific subclass of flavonoids in detriment of other, a separation from other phenolic and non-phenolic compounds, or even the isolation of a certain compound. Therefore, the nature of the matrix and the purpose of the extraction will define which extraction method will be applied.

Although there are some common criterions to guide its selection, in general, the best extraction method will be the one that, given the complexity of the biological matrix, will provide the highest extraction yields and avoid, as much as possible, the degradation of the compounds resulting from the different steps of the process.

1.1.2.2.1. Sample pre-treatment

The pre-treatment of the original material is frequently required before extraction. Initial steps of centrifugation and filtration may be performed. One other example is to submit the fresh material to drying processes to reduce the weight and volume of the sample, to minimize deteriorative biochemical reactions and inhibit microbial growth. When this step is performed by air-drying it may reduce the flavonoid content, as well as the antioxidant potential of the samples (41). As alternative, samples may be subjected to freeze-drying that efficiently removes water, and additionally modifies the physical microstructure of plant tissues leading to increased extraction yields (42). Other critical factor in the recovery of flavonoids is the particle size of the sample, since fine powders

improve the extraction by providing a large surface contact area between the solvent and sample (43). Therefore, milling, grinding or homogenization of plant material may be crucial steps before the extraction.

1.1.2.2.2. Extraction methods

Time of extraction, particle size, type of solvents, mass to volume *ratio*, and temperature are some of the variables that will determine the extraction yields. The extraction methods for flavonoids, likewise for or other bioactive compounds, are usually classified as conventional or unconventional. Conventional methods are those executed with simple and low-cost equipment that is at disposal in every laboratory, often require large amounts of solvents, and are time-consuming methods. In contrast, the unconventional methods are faster, use more expensive and sophisticated equipment, are more ecological, and are automated and reasonably reproducible processes. The conventional methods, such as decoction, maceration, infusion, digestion, and percolation, are based on liquid–liquid or solid–liquid extractions, of which times of extraction vary from a few minutes up to weeks. Despite their disadvantages related to the thermal degradation of compounds, the use of high amounts of non-environmentally friendly solvents and high energy consumption, decoctions and water infusions are conventional extractions still used today, Soxhlet extraction still being considered as a reference method (44). Flavonoids have a huge economical interest to the food, pharmaceutical and cosmetic industries. The growing interest in these compounds prompts continual search for green and economically feasible modern extraction methods (45). These unconventional extraction methods are broad group which includes mechanical force methods, electromagnetic forces, electrical forces, and enzymatic methods of extraction for specific biocatalytic reactions (46).

The extraction of flavonoids may be complemented with further processing steps, for instance if it is intended to fractionate the extract, to obtain a polyphenol-rich fraction or even to concentrate the sample. Several strategies, such as sequential extraction, liquid–liquid partitioning, or solid phase extraction (SPE) based on polarity and acidity, may be applied. Besides flavonoids, the extracts obtained from plants may contain different types of unwanted interferents (e.g., lipids, sugars, proteins organic acids or other phenolic compounds), thus it is crucial to remove them from the crude extracts prior to chemical profiling or assessment of their pharmacological potential. SPE is a helpful clean-up step of crude extracts, because it is a fast, reproducible, and low-cost procedure that can be applied to large or small extract volumes (47). Even through a simple SPE combining alkylated silica gels as sorbent (mainly C18) and solvents with distinct polarities, it is possible to fractionate

the crude extracts and obtain high flavonoid recoveries (48). To eliminate lipoid material the crude extract can be washed with non-polar solvents (e.g., hexane, dichloromethane, or chloroform), while other polar non-phenolic compounds and water-soluble compounds are washed away with acidified water (49). By the end, flavonoids are easily eluted with methanol. Further separation of phenolic compounds, or even of flavonoid sub-classes, can be achieved by adjusting the pH of the crude extract and the pH and polarity of eluents.

Other frequently required step after extraction, with relevance in the identification of flavonoids, is hydrolysis. Acidic hydrolysis is generally performed, although enzymatic hydrolysis is also an alternative (50). The flavonoids glycosides are hydrolysed with inorganic acids (the most common is HCl) at reflux, or above reflux temperatures, in aqueous or alcoholic solvents. The separation of the sugar moieties releases the flavonoid aglycones, thus helping to identify the compounds present in the extracts.

1.1.2.2.3. Analytical techniques

The most diversified analytical techniques are applied to identify flavonoids in the extracts. Chromatic reactions, such as the cyanidin reaction (51), the oxalo-boric reaction (52), the formation of aluminium-flavonoid chelates (53) or the vanillin-HCl reaction (54) are simple and fast screening methods. However, they often lack specificity, and frequently only allow the identification of some of the flavonoid sub-classes. This lack of specificity is a major drawback, especially in complex matrixes, which relegate such analytical methods in favour of others that allow the individual determination of flavonoids.

Liquid-chromatography (LC) is one of the most used analytical techniques in flavonoid analysis. Thin-layer chromatography (TLC) is relatively simple and low-cost, and widely used for the fingerprinting of natural extracts. Just like LC, it is based on the principle that the compounds in a mixture are separated due to their differential partition between the mobile and the stationary phases (55). Afterwards the chromatography plate is examined in ultraviolet-visible (UV-Vis) light, with or without derivatization, and compounds are identified by their retention factor values in comparison with the ones of reference substances, analysed under the same chromatographic conditions.

The improvement of LC hyphenated techniques, along with the development of new analytical software, made it possible in the same analysis, not only to separate the compounds, but also to individually identify and quantify them. The analysis of flavonoids by high-performance liquid chromatography (HPLC) was first implemented in 1976 by

Fisher and Wheaton, and ever since has become the analytical method of choice (56). In general, the analysis is performed with reversed-phase C18 columns ranging from 100 to 300 mm in length, and usually with a 4.6 mm internal diameter; the elution systems are usually binary, most likely acidified water and a less polar organic solvent, possibly acidified; each run does not exceed one hour; the flow rates are usually 1.0 or 1.5 mL/min; the columns are kept at ambient or slightly above ambient temperatures; the injection volume may range between 1 to 100 μ L (56). It is certain that specific modifications of these general conditions may be required due to the complexity of the sample, or to enhance chromatographic resolution.

Chromatographic hyphenated techniques combine the separation of compounds with on-line spectroscopic detection technology. The result is an overwhelming improvement of the capability of solving structural problems of complex natural products. The HPLC coupled to one, or several, spectroscopic detection techniques, such as diode array (DAD) or mass spectrometry (MS), has provided extremely powerful new tools that combine excellent separation efficiency with acquisition of spectroscopic data of a peak of interest within a complex mixture (57).

As phenolic compounds, flavonoids can be analysed by HPLC-DAD. The structure of the flavonoid effects on its chromatographic behaviour. Flavonoid aglycones are eluted later than the respective glycosides, the replacement of the hydroxyl-substituent with a methyl- or acetyl-substituent markedly increases the elution time, and the glycosylation position will affect the retention time (58). The slight structural modifications of their common structure are also reflected in distinct UV/vis absorption spectra. Thus, it is possible to classify a flavonoid in terms of the subclass to which it belongs. Furthermore, is possible to individually distinguish an individual flavonoid from others belonging to the same subclass.

Two major absorption bands are characteristic in flavonoids: band I (300–550 nm) corresponds to the B-ring and conjugation of the C-ring, the cinnamoyl system; band II (240–285 nm) corresponds to the A-ring, the benzoyl system (50). In general, simple substituents, such as methyl, methoxy and non-dissociated hydroxyl groups generally effect only minor changes in the position of the absorption maxima, and acetylation tends to nullify the effect of the phenolic hydroxyl groups on the spectra (59).

The absence of a 3-hydroxyl group in flavones (band I absorption between 304–350 nm) distinguishes them from flavonols (between 352–385 nm). The increasing oxygenation of flavones and flavonols tends to slightly increase the band I absorption maxima, as observed for the shift to 359 nm in galangin (3,5,7-triOH), to 367 nm in kaempferol (3,5,7,4'-tetraOH), to 370 nm in quercetin (3,5,7,3',4'-penta OH) and to 374 nm

in myricetin (3,5,7,3',4',5'-hexaOH) (60). On the other hand, band II is significantly affected by changes of the oxygenation pattern of the A-ring, it appears as one peak (270 nm) in compounds with a monosubstituted ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substitution is B present (60).

Flavanones and dihydroflavonols have a saturated heterocyclic C-ring with no conjugation between the A- and B- rings (**Figure 2**). Flavanones and dihydroflavonols normally exhibit a very strong band II absorption maximum between 270–295 nm, and only a shoulder for band I at 326–327 nm. In isoflavones, band II appears in the 245–270 nm region and band I shoulder in the range of 300–340 nm. The oxygenation and substitution patterns in B-ring do not largely affect their spectra, but the increased A-ring oxygenation leads to a bathochromic shift in the band II absorption (61).

Anthocyanidins (**Figure 2**) exhibit a band I peak in the 450–560 nm region due to the hydroxyl cinnamoyl system of the B-ring, the band II is represented by a less intense peak in the 270–280 nm region, and their colours vary with the number and position of the hydroxyl groups (61).

Chalcones and aurones (**Figure 2**) are characterized by the presence of a dominant band I absorption and a relatively minor band II in their UV spectra. Chalcones exhibit band II in the 220–270 nm region and band I usually within the range of 340–390 nm. Methylation or glycosylation has little effect on their spectrum unless it occurs at the 2'-position that results in a hypsochromic shift. In aurones band I is generally found between 370–430 nm (60), and the substitutions at 7-position in 6,7 -dihydroxyaurones result in an hypsochromic shift of band I.

The qualitative analysis of flavonoids by HPLC–DAD may not be enough to provide concise data, and nowadays is primarily used for quantification by interpolation of external calibration curves. In terms of quantitative analysis, the typical wavelength for the quantification of flavanones is between 280 nm and 290 nm, flavones and flavonols are quantified between 350 and 370 nm, isoflavones and catechins at 280 nm, anthocyanins in the range 502–525 nm, aurones in the range 370–430 nm, and chalcones in the range 340–390 nm (62).

LC-MS is the state-of-the-art detection in the qualitative analysis of flavonoids. Nevertheless, because UV–vis detection is non-destructive, this detector can be connected before others and is frequent its combination with MS to facilitate the identification of flavonoids. The combination of these hyphenated techniques has applicability in the identification of several glycosylated flavonoids, which exhibit identical molecular formulae

and fragmentation patterns, yet belonging to different flavonoid sub-classes (63). The combination of chromatographic resolution with the structural information provided by MS^n enables routine separation and tentative identification of flavonoids in complex mixtures.

The general ionization and fragmentation of flavonoids is largely independent of the ionisation source, although some variations of the relative ion intensities may be observed. Electrospray ionization (ESI) consists in the generation of ions by solvent evaporation under a high voltage potential. In flavonoid analysis, either negative or positive ionization is adequate. However, negative ionization has been shown to be more sensitive, as consequence of the increased background noise in positive ionization mode (63). Structural information obtainable from the mass spectra may be achieved when tandem mass spectrometric techniques are applied, for instance when ESI interface is coupled to a quadrupole time-of-flight (QTOF) mass spectrometer. By using an ion-trap instrument like QTOF it is possible to perform MS^n experiments, and tandem-MS detection has largely replaced single-stage MS because it enables the confirmation of proposed reaction pathways for fragmentation ions.

In first-order MS spectra of flavonoids de-protonated $[M-H]^-$ ions are the most common, with little fragmentation present. A widely accepted fragment nomenclature is used to designate fragment ions yielded by mass spectrometric analysis of flavonoids (64). For free aglycones, the labels $^{ij}A_o$ and $^{ij}B_o$ respectively designate the fragments containing intact A- and B-rings, the superscripts i and j indicate the broken C-ring bonds, and the ions can be positive or negative depending on the ionization mode (superscript + or -). The subscript o distinguishes these ions from carbohydrate fragments $^{k,l}A_i^-$ and B_i^- ($i \geq 1$). The fragmentation pathways of the C-ring occur at typical bond positions (**Figure 5**) and entail retro-Diels–Alder reactions. The formed fragmentation ions provide valuable information on the number and nature of substituents on A- and B-rings.

In terms of general fragmentation patterns in negative mode, the $^{1,3}A_o^-$ ion is observed for all flavonoid sub-classes, is most readily formed and often constitutes the base peak ion in second-order mass spectra; for flavones and flavonols, cleavage of the $o/2$ bonds is common leading to the formation of the $^{o,2}B_o^-$ ion, while the corresponding $^{o,2}A_o^-$ ion is characteristic for flavonols; further losses of 18 amu (H_2O), 28 amu (CO), 42 amu (C_2H_2O) and 34 amu (CO_2) or successive losses of these groups are also commonly observed in second-order mass spectra; in the case of *O*-methylated flavonoids the loss of 15 amu (CH_3) is prominent, the $[M-H-CH_3]^-$ radical ion generally constituting the base peak (63).

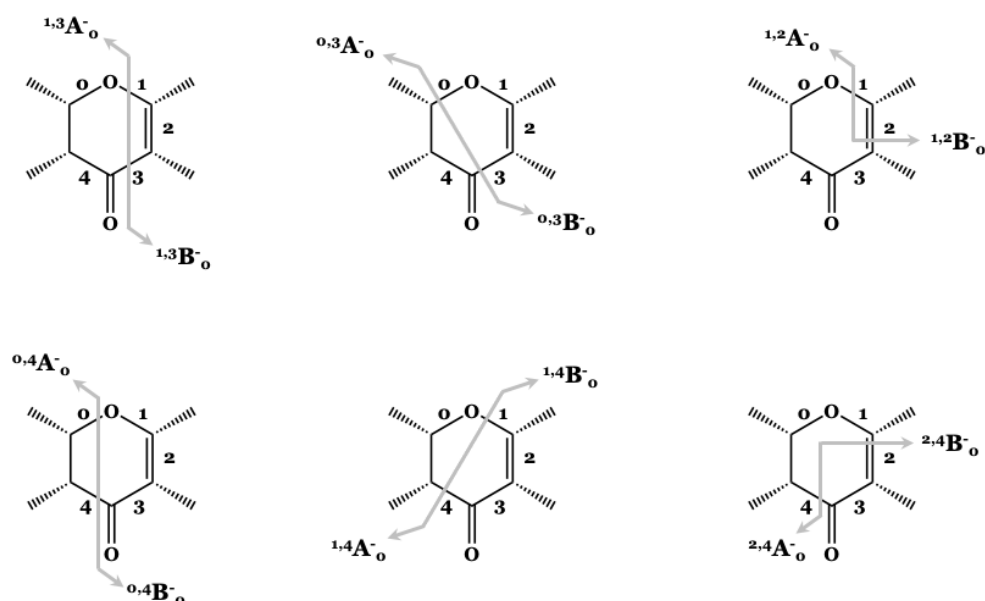


Figure 5. Nomenclature of fragmentation ions used in mass spectrometry analysis of flavonoid aglycones, and fragmentation by cleavage of C-ring bonds in negative ionization mode (adapted from (59)).

In proanthocyanidins, the elimination of a B-ring because of retro-Diels-Alder fission of C-ring is favoured in the top unit and is commonly followed by the loss of an additional water molecule; a second pathway, designated by heterolytic ring fission, involves the loss of the A-ring, again primarily of the top unit ($^{1,4}\text{B}^-$ fragment); the third fragmentation pathway involves quinone methide cleavage of the interflavan bond (63).

As previously mentioned, flavonoids are also found as glycosidic species, two types being distinguished based on the nature of the flavonoid-saccharide linkage (*O*-glycosides and *C*-glycosides). The fragment nomenclature of flavonoid-glycosides designates that when the charge is located on the aglycone fragment (superscript + or – for positive or negative ions, respectively) the ions are labelled as $^{k,l}\text{X}^{\pm}_j$, $\text{Y}^{\pm}_j$ and $\text{Z}^{\pm}_j$, the subscript j denotes the number of the interglycosidic bond cleaved counted from the aglycone (the aglycone-glycosidic bond is numbered 0), and superscripts k and l indicate cleavages within the carbohydrate rings (**Figure 6**) (63). In the *C,O*-glycosides, *O*-glycosylated on the *C*-glycosyl moiety, the first glycosidic bond does not connect to the aglycone, thus the numbering starts at the *C*-glycosyl moiety. In addition, in this situation the Y_0 or Z_0 ions represent fragments resulting from the loss of the *C*-glycosyl moiety (64). If more than one glycosylation is involved, an additional superscript m is used to denote the position of the other substitution (e.g., ion $^{k,l}\text{X}^-_{mj}$). In the case of charged carbohydrate fragments, ions are designated by $^{k,l}\text{A}^{\pm}_i$, $\text{B}^{\pm}_i$ and $\text{C}^{\pm}_i$, and subscript i (≥ 1) represents the number of the glycosidic bond cleaved counting from the non-reducing end numbered as 1 (**Figure 6**) (63).

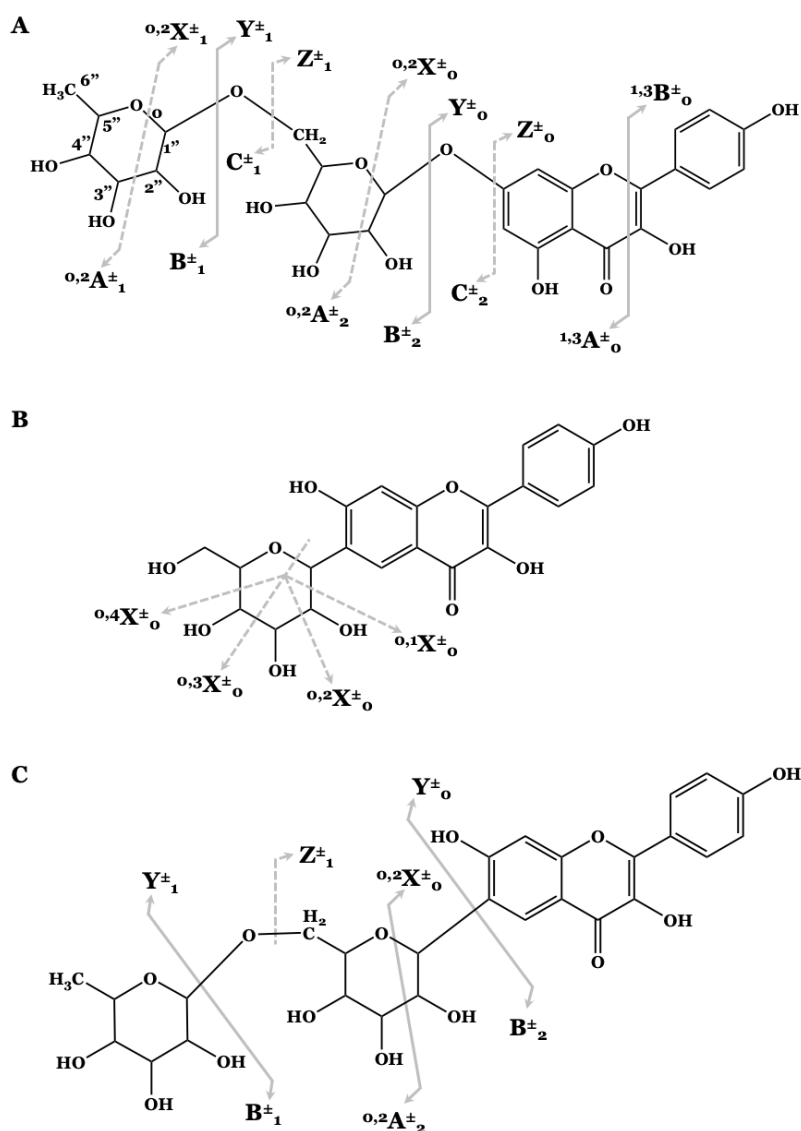


Figure 6. Nomenclature of fragmentation ions used in mass spectrometry analysis of flavonoid-O-glycosides (A), flavonoid-C-glycosides (B) and flavonoid-C,O-glycosides (C), and general fragmentation schemes (adapted from (63,64)).

In terms of fragmentation patterns, by the analysis of the resulting fragments is possible to characterize the glycosylation type (O-, C-, or C,O-glycosides) and its position (e.g., 3-O-glycoside or 7-O-glycoside); it is also possible to determine the glycoside type (e.g., hexoses, deoxyhexoses, and pentoses), its distribution (e.g., mono-, di- or tri-glycosylation), its sequence, and its interglycosidic linkage (64).

Overall, the identification of compounds may be achieved by comparing the experimental DAD and MS data, preferentially with that from external standards analysed under the same chromatographic conditions. The identification is also possible in resort to a range of electronic databases.

1.1.2.3. Impact on human health

The flavonol rutin, isolated from citrus fruit, was first classified as a compound from a new class of vitamins and designated as “vitamin P” (65). Since then, the advances of the scientific knowledge on flavonoids, not only helped to understand their biosynthesis and roles in plants, but also their impact on human health. The number of published papers *per year* on flavonoids has risen from 740 in 1991 to more than 9000 in 2015, overall reaching nearly 90 000 (65).

Flavonoids are abundantly present in foods and beverages of plant origin; thus, they are often termed as dietary flavonoids and “nutraceuticals”. The estimation of flavonoid intake in populations is important for epidemiological studies of their health benefits. The Third National Health and Nutrition Examination Survey, conducted in the USA (1988-1994), evaluated the health and nutritional status of the population. It was reported that tea, wine, beer, citrus fruits and apples were the most important sources of total flavonoid intakes, and it was estimated that the mean total flavonoid intake by US adults was 344.83 ± 9.13 mg/day; flavan-3-ols were the most abundant sub-class (191.99 ± 6.84 mg/day) followed by proanthocyanidins (98.29 ± 8.31 mg), flavanones (22.38 ± 1.79 mg/day), flavonols (18.09 ± 0.82 mg/day), anthocyanidins (9.20 ± 0.79 mg/day), isoflavones (2.35 ± 0.27 mg/day), and flavones (1.38 ± 0.09 mg/day) (66). In a latter study, it was reported that the total flavonoid intake of USA adults aged 19 years and older remained unchanged between 1999-2002 (201.9 mg/day) and 2007-2010 (200.1 mg/day), with tea as the top source of flavonoids (67).

In a study conducted in 14 European countries, between 1999 and 2005, was estimated that the total flavonoid intake for the age group of 18–64 years-old adults was 428 ± 49 mg/day, of which the flavan-3-ols gallic acid derivatives were the main contributors (53 ± 12 mg/day); the highest intake was in the Central Region (506 mg/day), followed by the Northern Region (348 mg/day) and the Southern Region (301 mg/day) (68).

The relationship between long-term consumption of flavonoids and its benefits to human health has been addressed by several epidemiological studies, which were triggered by the hypothetical inverse associations of flavonoid intake and major causes of death in Western countries. The use of meta-analyses to synthesise the evidence from epidemiological studies has led to a more comprehensive overview of the potential beneficial impact of flavonoids on human health. A meta-analysis of randomized controlled trials reported clinically relevant effects of some flavonoid sub-classes or flavonoid-rich foods (e.g., chocolate, cocoa, and black tea) on cardiovascular disease risk factors (69).

The moderate habitual intake of flavonoids was also inversely associated with all-cause, cardiovascular- and cancer-related mortality, with strong association plateaus at intakes of approximately 500 mg/day (70). It was also reported that the established protective effects of fruit and vegetables against gastric cancer can, in part, be due to the presence of flavonoids (71).

Other meta-analysis of prospective cohort studies implied that an increase in the total flavonoids intake of 500 mg/day was associated with a significant risk reduction of type 2 diabetes (72).

A significant inverse association between higher dietary intake of total flavonoids (sub-classes of flavan-3-ols, anthocyanins and flavonols, and quercetin individually) and impaired cognitive status was observed among the adult population living in the Mediterranean area (73).

Regarding neurodegenerative disorders, the higher long-term dietary intakes of flavonols, anthocyanins, and flavonoid polymers are associated with lower risks of Alzheimer's disease (AD) and AD-related dementias (74,75). According to longitudinal studies, the habitual consumption of green tea (abundantly containing catechin and their gallate derivatives and polymers) is inversely correlated with the onset of Parkinson's disease (PD) (76). Epidemiological studies have provided that dietary consumption of berries and herbs with high flavonoid content can reduce the risk of PD (77).

The pleiotropic beneficial effects of flavonoids are limited by their bioavailability. This is an inevitable key determinant since there is a significant divergence between the total flavonoid content in diet and the blood concentrations reached after their consumption. Despite their abundance in human diet, it is estimated that their oral bioavailability is less than 10% (78). The oral bioavailability is determined by the physicochemical characteristics of each flavonoid (e.g., molecular weight, lipophilicity, glycosylation, conjugation, and degree of polymerization) (79), and by the wide interindividual variability in flavonoid absorption and metabolism (e.g., diet, age, sex, genotype, and prescribed medication) (80).

In a general overview, after ingestion, the absorption of flavonoids takes place in the lumen of the small intestine (duodenum and jejunum). Flavonoid glycosides are cleaved before absorption. The deglycosylation is mediated by lactase-phlorizin hydrolase, which is found in the brush border portion of intestinal epithelial cells. As result of the increased lipophilicity, free aglycones may enter the epithelial cells by passive diffusion. In alternative, flavonoid glycosides can be directly transported into the epithelium *via* transporters (e.g., P-glycoprotein (P-gp) and sodium-dependent glucose transporter (SGLT)), and then

hydrolysed by intracellular enzymes (e.g., β -glucosidases) (81). The flavonoids that are not absorbed in the small intestine reach the large intestine, where they are degraded by colonic microflora (82).

Once absorbed, flavonoids are metabolized in enterocytes, and in great extent in the liver. The hepatic metabolism may occur by phase I reactions (e.g., hydroxylation catalysed by cytochrome P450 (CYP450)), by phase II reactions resulting in sulphate, glucuronide and/or methylated metabolites through the respective action of the conjugation enzymes sulfotransferases, UDP-glucuronosyltransferases (UGTs) and catechol-O-methyltransferase (COMT) (83). The higher hydrophilicity of the metabolites facilitates their elimination. The flavonoid metabolites that re-enter the enterohepatic circulation through bile excretion are hydrolysed by the microbiota to aglycones, and further catabolized to low molecular weight phenolic compounds that can readily be absorbed (82).

Notwithstanding the difficulties of assessing their bioavailability, the increasing knowledge already evidences some differences between sub-classes: isoflavones show the highest bioavailability of all sub-classes of flavonoids and it does not differ between their aglycones and glucosides; the bioavailability of flavanones and flavonols is reduced when disaccharides are linked to the aglycones; catechins are rapidly absorbed and the bioavailability of the various catechin monomers seems to be similar, but dimerization reduces the bioavailability; the bioavailability of anthocyanins seems to be the lowest of all flavonoids (84).

The consumption of polyphenol-dietary supplements may have a positive outcome, but, at the same time, it may be a double-edge sword. Flavonoid-rich supplements are generally regarded as safe, based on the long history of use. However, the consumption of such products can increase dietary flavonoid intake, and subsequently plasma concentrations beyond expected levels. Therefore, it has been hypothesised that these high doses can mechanistically cause adverse effects through pro-oxidative action or even by interaction with other pharmacologic agents (e.g., herb-drug interactions) (85).

For some time, flavonoids were considered merely as active components of medicinal plants. Nowadays, they switched to be indispensable components in a variety of nutraceutical, pharmaceutical, medicinal, and cosmetic applications, and it is envisioned that the increasing knowledge on flavonoids will continuously demonstrate their importance for human health (86)

1.2. Flavonoids and the Central Nervous System

1.2.1. Overview of aging-associated and chronic disorders

Roman Terentius claimed “*Senectus ipsa est morbus*” (old age itself is a disease), and, in fact, aging is the predominant risk factor for most diseases that limit healthspan (87). The population in the European Union is older than the global population, and that suggests that it might be particularly vulnerable to an increasing burden of age-related neurological disorders. In 2017, neurological disorders ranked third after cardiovascular diseases and cancer representing 19.5% of total deaths. During the study period (1990–2017) was found a substantial increase in the all-age burden of neurodegenerative disorders, which was higher in men than in women and peaked in individuals aged 80–84 years (88).

Neurodegenerative disorders tend to progress in an irreversible manner and are closely associated with the nine biological hallmarks of ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence, deregulated nutrient sensing, stem cell exhaustion, and altered intercellular communication (89). They manifest by many different symptoms (e.g., cognitive impairment, speech difficulties and motor dysfunction), but despite the specific pathological hallmarks of each one, they all share the loss of neuronal populations in the brain and/or spinal cord (90). The clinical features of a given neurodegenerative disorder are dictated by the specific areas of the brain and spinal cord in which neuronal loss occurs. In this broad group are included more common disorders, such as AD, PD, amyotrophic lateral sclerosis, and Huntington’s disease (HD), and others less common (dementia with Lewy bodies, progressive supranuclear palsy, multiple system atrophy, corticobasal degeneration, frontotemporal dementia, spinocerebellar ataxia disorders, and spinal muscular atrophy, among others) (90).

AD is the leading cause of dementia (accounting for 50–70% of cases) and its prevalence is increasing dramatically worldwide with the ageing of populations. In 2015, it was estimated that almost 47 million people were affected by dementia (91). This disorder has a huge economic impact. In 2011, a multinational study (including data from Spain, Sweden, UK, and USA) estimated that societal costs amount to about €14,500 per year in patients at home, rising to €72,500 per year in patients who need residential care (92). In AD the neuronal loss involves the cortical and hippocampal regions, and this disorder is pathologically characterized by amyloid- β (A β) accumulation, neurofibrillary tangle formation, extensive neuroinflammation, synaptic toxicity, neurodegeneration, and brain

dysfunction, patients presenting memory impairment (93). Among the currently available pharmacotherapy are acetylcholinesterase inhibitors (e.g., rivastigmine, galantamine, donepezil) and N-methyl-D-aspartate (NMDA) receptor antagonists (memantine) (94).

PD is also a progressive neurodegenerative disorder, classically classified as a motor disorder. In 2016, it was estimated that 6.1 million individuals had PD worldwide (95). As the second most common neurodegenerative disorder after AD, it also has a significant economic impact. In Portugal alone, by 2011, it was estimated a direct cost of 2,717 €/patient for a six-month period (96). PD is marked by a progressive degeneration of the nigrostriatal dopaminergic pathway with substantial loss of substantia nigra pars compacta neurons and depletion of dopamine (DA). In addition, the increased alpha-synuclein accumulation, mitochondrial dysfunction, oxidative stress, excitotoxicity, and neuroinflammation also play a crucial role in the pathogenesis of PD (97). The primary manifestations include dysfunctions of the somatomotor system (i.e., rigidity, bradykinesia, postural instability, and tremors); other non-motor symptoms including neuropsychiatric symptoms (depression, cognitive dysfunctions, and dementia), sleep disorders (insomnia, rapid eye movement disorders, vivid dreaming) and autonomic symptoms (bladder disorders, orthostatic hypotension, erectile impotence) can occur during disease course (98). The current pharmacological treatment of PD is based on the replacement of DA using DA precursors (3,4-dihydroxy-L-phenylalanine (L-DOPA)), DA agonists (e.g., amantadine and apomorphine) and monoamine oxidase-B (MAO-B) inhibitors (e.g., selegiline and rasagiline), which can be used alone or in combination (99).

Despite the significant scientific advances, in this field, the current pharmacotherapy is directed to the treatment of the symptoms of neurodegenerative disorders. Therefore, new neuroprotective strategies are needed to slow down, or even to stop, the neurodegenerative process along with symptomatic treatment, even if they are not curative or able to restore those cells already lost by the time of diagnosis (90).

1.2.2. Flavonoid-based therapies

The application of flavonoid-based therapies in the early management of neurodegenerative disorders is highlighted as possible alternative strategy to prevent or manage the progression of neurodegenerative disorders (100). Unfortunately, the pleiotropic effects of flavonoid-based therapies are not always translated into clinical evidence, possibly because pre-clinical models fail to fulfil their predictive purpose, or because clinical trials may be incapable of detecting the beneficial effects due to a defective

approach (75,101). A few clinical trials have been designed to highlight general neuroprotective effects by using flavonoids, flavonoid rich-extracts, or formulations, from which positive results were associated with acute improvements in cognitive function and enhanced blood flow (102), and increased *N*-acetyl-aspartate, a marker of neuronal metabolic health (103).

The interest in flavonoids is related with their wide target versatility that leads them to trigger multiple responses, and their safety profile (104). A substantial body of scientific data demonstrates the neuroprotective effects of flavonoids in pre-clinical models of AD (**Table 1**) and PD (**Table 2**). Altogether, these findings strongly support that flavonoids act on several different pathophysiological changes that occur in the aging brain and that are exacerbated in AD and PD. Flavonoids enhance cognitive function and modulate behavioural impairments, and increase brain cell function and neuronal survival by reducing oxidative stress, modulation of neurotrophic factor signalling pathways, prevention of alterations in protein processing, reducing synaptic dysfunction and inhibition of inflammatory response (105). Therefore, flavonoid-based therapies may have potential in the prevention or treatment of these neurodegenerative disorders.

The individual effect of a particular flavonoid is obviously easier to interpret than that of a flavonoid-rich extract. The predictions cannot be based on the mechanisms exerted by a single flavonoid, instead they may result from additive, synergism, or antagonism effects. However, the study of flavonoid-based therapies also includes flavonoid-rich extracts obtained from medicinal plants in pre-clinical models of AD (106–109) and PD (110–112).

Table 1. Neuroprotective effects of flavonoids in pre-clinical models of Alzheimer's disease.

	Animal model	Treatment	Proposed mechanism of action
Flavones (ref.)			
Apigenin (106)	APP/PS1 mice	Oral gavage 40 mg/kg/day, 12 weeks	Amelioration of learning and memory impairment by suppressing the amyloidogenic process, and by inhibiting oxidative stress and restoring ERK/CREB/BDNF pathway
Baicalein (107)	Female Tg2576 mice	Intraperitoneal 10 mg/kg/day, 8 weeks	Enhanced APP α -secretase processing, reduced A β production and improved cognitive performance
Nobiletin (113–115)	TgAPP-SL 7-5 mice	Intraperitoneal 10 mg/kg/day, 4 months	Decreased A β burden in the hippocampus
	Male 3 $\times$ Tg-AD mice	Intraperitoneal 30 mg/kg/day, 3 months	Reversed impairment of short-term memory and recognition memory, and reduction of soluble A β and ROS levels in the hippocampus
	Male SAMP8 mice	Intraperitoneal 10 and 50 mg/kg/day, 4–6 months	Reversed impairment of recognition memory and context-dependent fear memory, reduced oxidative burden, and reduced tau phosphorylation in the hippocampus
Tropoflavin (116,117)	5 $\times$ FAD mice	Intraperitoneal 5 mg/kg, once daily, 10 days	Restored TrkB signalling without affecting endogenous BDNF levels, and decreased BACE1 expression
Flavanones (ref.)			
Hesperidin (118)	Male APP/PS1	Oral gavage 100 mg/kg/once daily, 10 days	Significant attenuation of β -amyloid deposition, microglial activation and TGF- β 1

Table 1. Cont.

	Animal model	Treatment	Proposed mechanism of action
Flavan-3-ols (ref.)			
3'-O-Methyl-epicatechin-5-O- β -glucuronide (108)	Female Tg2576 mice	Drinking water 80 mg/kg/day, 5 months	Promotion of basal synaptic transmission and long-term potentiation in hippocampus associated with CREB signalling
Epigallocatechin gallate (109,110)	TgCRND8 mice	Drinking water 50 mg/kg/day, 4 months	Lower levels of soluble A β 1-42 in the cortex and hippocampus
	SAMP8 mice	Drinking water 15 mg/kg/4 h a day, 8 weeks	Reduced levels of A β 1-42 and BACE-1 in frontal cortex and hippocampus. Prevention of tau hyperphosphorylation, and reversion of decreased synaptic protein markers and postsynaptic density proteins in frontal cortex and hippocampus
Flavonols (ref.)			
Fisetin (111,112,119)	APPswe/PS1dE9 mice	Fat diet 0.05% fisetin, 8 months	Increase in ERK phosphorylation along with a decrease in protein carbonylation
	Male SAMP8 mice	Diet 25 mg/kg/day, 7 months	Restore of multiple markers associated with impaired synaptic function, stress, and inflammation
	Male C57BL/6N mice treated with A β 1-42	Intraperitoneal (20 mg/kg/day, 2 weeks)	Decreased accumulation of A β , and decreased BACE-1 expression and tau hyperphosphorylation. Reversed synaptic dysfunction by increasing the levels of both presynaptic and postsynaptic proteins. Prevention of neuroinflammation
Quercetin (120)	3 $\times$ Tg-AD mice	Intraperitoneal 25 mg/kg every 48 h, 3 months	Decreased extracellular β -amyloidosis, tauopathy, astrogliosis and microgliosis in the hippocampus and the amygdala
Quercetin and Kaempferol (121)	Male TgAPPswe/PS1e9 mice	Oral gavage 50 mg/kg, once daily, 4 months	Stimulation of depression-related signalling pathways involving BDNF/CREB/PSD95. Reduction of A β peptides in hippocampus

Table 1. *Cont.*

	Animal model	Treatment	Proposed mechanism of action
Flavonols (ref.)			
Quercetin-3- <i>O</i> -glucuronide (122)	Male Tg2576 mice	Intragastric gavage 150 mg/kg once daily, 10 days	Significant reduction of A β peptides, and improvement in hippocampal basal synaptic transmission and long-term potentiation
Quercetin-3- <i>O</i> -rutinoside (123)	Male Wistar rats treated with A β 1–42	Intraperitoneal 100 mg/kg, once daily, 3 weeks	Significant increase of ERK1, CREB and BDNF gene expression in the hippocampus of rats, and decreased oxidative burden

A β , amyloid- β peptide; APP, amyloid precursor protein; BACE, β -secretase enzyme; BDNF, brain-derived neurotrophic factor; CREB, cyclic AMP response element binding protein; ERK, extracellular signal-regulated kinases; PHF, paired helical filament; PSD, postsynaptic density proteins; ROS, reactive oxygen species; TGF- β 1, anti-transforming growth factor beta 1; TrkB, tyrosine receptor kinase B.

Table 2. Neuroprotective effects of flavonoids in pre-clinical models of Parkinson's disease.

	Animal model	Treatment	Proposed mechanism of action
Flavones (ref.)			
Apigenin (124,125)	Male Sprague-Dawley rats treated with rotenone	Intraperitoneal 10 and 20 mg/kg/day, 14 days	Improved mitochondrial activity. Attenuation of the upregulation of NF- κ B gene expression and prevention of neuroinflammation in SNpc. Downregulated α -synuclein aggregation, and upregulated TH protein and DA-D2 receptor expressions
	Male Swiss-albino mice treated with MPTP	<i>Per os</i> 5, 10 and 20 mg/kg/day, 26 days	Improved locomotor and muscular activities. TH-positive cells protected from degeneration. Decreased GFAP levels in the SN, and elevated BDNF levels
Baicalein (126–128)	Male Sprague-Dawley rats treated with MPP ⁺	Intraperitoneal 30 mg/kg/day, 7 days	Attenuation of striatal DA content reduction and of TH. Reduced α -synuclein aggregates, ED-1, activated caspase-1, IL-1 β and cathepsin B
	Male C57BL/6 mice treated with MPTP	Intragastric 200 mg/kg/day, 7 days	Increased levels of DA and serotonin in the striatum by increasing the counts of dopaminergic neurons, inhibition of oxidative stress and astroglia response
	Male Sprague-Dawley rats treated with rotenone	<i>Per os</i> 100–400 mg/kg/day, 30 days	Attenuation of dopaminergic neuron depletion, restored mitochondrial function, and improved mitobiogenesis
Chrysin (129)	Male C57B/6J mice treated with 6-OHDA	Oral administration 10 mg/kg/day, 28 days	Modulation of inflammatory cytokines and neurotrophic factors, and recovery of dopaminergic neurons in striatum
Luteolin (125)	Male Swiss-albino mice treated with MPTP	Oral administration 10 and 20 mg/kg/day, 26 days	Improved locomotor and muscular activities. TH-positive cells protected from degeneration. Decreased levels of GFAP in the SN, and elevated BDNF levels
Morin (130)	Male C57BL/6 mice treated with MPTP	Intraperitoneal 50 mg/kg/day, 12 days	Modulation of mitochondrial dysfunction and ROS production, and reduction of the activation of astroglia and inflammation

Table 2. *Cont.*

	Animal model	Treatment	Proposed mechanism of action
Flavones (ref.)			
Nobiletin (131)	Female Sprague-Dawley rats treated with MPP ⁺	Intraperitoneal 10 mg/kg/day, 7 days	Protection of dopaminergic neurons in the SN and inhibition of microglial activation
Tropoflavin (132–135)	Male Sprague-Dawley rats treated with 6-OHDA	Drinking water 12–16 mg/kg/day, 4 weeks	Prevented the loss of dopaminergic neurons in SN
	C57BL/6 mice treated with MPTP	Intraperitoneal 5 mg/kg/day, 14 days	Reduced loss of dopaminergic neurons in the SN and striatum and reduced oxidative burden
	Male C57BL/6J mice treated with MPTP	Intraperitoneal 5 mg/kg/day, 4 weeks	TH levels were maintained. Increased levels of SCG10, phosphorylated TrkB and phosphorylated ERK1/2
	Cynomolgus monkeys treated with MPP ⁺	Oral administration 30 mg/kg/day, 7 months	Prevention of progressive degeneration of midbrain dopaminergic neurons
Flavan-3-ols (ref.)			
Catechin (136)	Male Wistar rats treated with 6-OHDA	Intraperitoneal 10 and 30 mg/kg/day, 14 days	Antioxidant action, increased DA content and TH
Epicatechin (137)	Male Wistar rats treated with MPP ⁺	<i>Per os</i> 100 mg/kg/day, 5 days	Increased Cu/Zn-superoxide dismutase activity
Epigallocatechin gallate (138)	Male C57BL/6 mice treated with MPTP	<i>Per os</i> 25 mg/kg/day, 7 days	Regulation of ferroportin in SN and reduced oxidative stress
Flavanones (ref.)			
Hesperetin (139)	Male Wistar rats treated with 6-OHDA/AA	<i>Per os</i> 50 mg/kg/day, 1 week	Attenuation of striatal oxidative burden, lowered striatal level of glial fibrillary acidic protein and increased Bcl2

Table 2. *Cont.*

	Animal model	Treatment	Proposed mechanism of action
Flavanones (ref.)			
Hesperidin (140)	Female C57 BL/6 mice treated with 6-OHDA	<i>Per os</i> 50 mg/kg/day, 28 days	Prevention of memory impairment in the Morris water maze test, as well as depressive-like behaviour in the tail suspension test; antioxidant mechanisms
Naringenin (141,142)	Male C57BL/6J mice treated with MPTP	<i>Per os</i> 25, 50, and 100 mg/kg/day, 5 days	Downregulation of α -synuclein, and upregulation of DA transporter and TH expressions. Reduced neuroinflammation and oxidative stress
	Male Sprague-Dawley rats treated with 6-OHDA	<i>Per os</i> 50 mg/kg/day, 4 days	Protection of TH-positive cells in the SN, and maintenance of the striatal DA levels
Naringin (143)	C57BL/6 mice treated with 6-OHDA/AA	Intraperitoneal 80mg/kg/day, 6 days	Activation of mammalian target of rapamycin complex 1 and inhibition of microglial activation in the SN
Flavonols (ref.)			
Kaempferol (144)	Male C57BL/6 mice treated with MPTP	Oral administration 25, 50 and 100 mg/kg/day, 14 days	Improved motor coordination, raised striatal DA levels, reduced oxidative burden and prevention of TH-positive neuron loss
Quercetin (145–147)	Sprague-Dawley rats treated with rotenone	Intraperitoneal 50 mg/kg/day, 4 days	Complete reversion of striatal dopaminergic loss and nigral glutathione depletion. Reduced rotenone-induced behavioural changes and loss of TH immunoreactivity in both the SN and striatum
	Male C57BL/6 mice treated with MPTP	Oral administration 100 and 200 mg/kg/day, 14 days	Significant increased activities of antioxidant enzymes and acetylcholinesterase, and significant decrease in markers of lipid peroxidation
	MitoPark mice	Oral gavage 25 mg/kg/day, 6 weeks	Reversion of behavioural deficits, of striatal DA depletion and of TH neuronal cell loss

Table 2. *Cont.*

	Animal model	Treatment	Proposed mechanism of action
Flavonols (ref.)			
Quercetin-3-O-rutinoside (148)	Male Wistar rats treated with 6-OHDA/AA	Oral administration 25 mg/kg/day, 3 weeks	Reduction of oxidative burden and significant increase in striatal DA levels

6-OHDA, 6-hydroxydopamine; AA, ascorbic acid; Bcl2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; CREB, cyclic AMP response element binding protein; DA, dopamine; ED-1, biomarker for microglia activation; GFAP, glial fibrillary acidic protein; IL, interleukin, MPP⁺, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; SCG-10, superior cervical ganglion; SN, substantia nigra; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TrkB, tyrosine receptor kinase B.

1.2.3. Effects in common targets and pathways underpinning neurodegeneration

Flavonoids are considered to be multiplayers since they interact with multiple common targets and processes underpinning neurodegeneration. They have the potential to balance oxidative stress, to prevent alterations in protein processing, to decrease neurotrophic factor signalling, synaptic dysfunction and neuroinflammation, and have marked effects on the attenuation of behavioural impairments and cognitive dysfunction (105,149–151). Nowadays, it is hypothesized that the strategies to prevent, halt, or delay neurodegenerative disorders may profit from an integrative combination based on a multitarget therapeutic approach (152).

1.2.3.1. Neuroprotective signalling pathways mediated by flavonoids

Flavonoids are found *in vivo* in plenty concentrations to act as signalling molecules likely to profoundly affect cellular functions, but their precise sites of action are still unknown. It is likely that their activity depends on their ability to bind to ATP binding sites on enzymes and receptors, to directly modulate the activity of kinases, to affect the function of important phosphatases, to preserve neuronal calcium homeostasis, and to modulate signalling cascades lying downstream of kinases (i.e., transcription factor activation and binding to promoter sequences) (151,153,154).

They exert neuroprotective functions through interaction with proteins involved in protein kinase and lipid kinase signalling cascades that are associated with cellular survival and apoptosis, such as those of phosphatidylinositol 3-kinase (PI-3K), Akt/protein kinase B (Akt/PKB), tyrosine kinase, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs; stress-activated protein kinases/Jun amino-terminal kinases (JNK/SAPK), and extracellular signal-regulated kinase (ERK) and p38 (154). In general, the activation of ERK, Akt/PKB, PI-3K, and PKC pathways is important to improve cell's survival. Conversely, the downregulation of P38 and JNK/SAPK prevents apoptosis (154).

By affecting signalling pathways, flavonoids also have influence on learning, memory, and cognition. They are well known modulators of neuronal signalling pathways in the context of long-term potentiation, synaptic plasticity, and memory, such as cyclic adenosine monophosphate (cAMP)-dependent protein kinase, PKB/Akt, PKC, calcium calmodulin kinase (CaMK) and ERK, all these five pathways converge to signal to the cAMP-response element-binding protein (CREB) (155). CREB is a transcription factor that is activated by

neuronal activity, and it is a key regulator of an increasingly complex biochemical pathway (CREB isoforms function either as activators of gene expression or as repressors of the activators) that produces changes in the strength and structure of synaptic connections between neurons with associated synapse re-modelling, synaptic plasticity, and memory (156). Among all these pathways, flavonoids appear to interact primarily with the ERK and PKB/Akt pathways (154). Of the several molecules that are regulated by CREB, the brain-derived neurotrophic factor (BDNF) is a neurotrophin known to be required during memory acquisition and consolidation (156). Furthermore, flavonoids may exert effects on endothelial function and peripheral blood flow, and these vascular effects are potentially significant as increased cerebrovascular function is known to facilitate adult neurogenesis and influence memory (150,155).

1.2.3.2. Flavonoids in the balance of oxidative stress

Oxidative stress is the result of an imbalance in pro-oxidant/antioxidant cellular homeostasis that leads to the overproduction of toxic reactive oxygen species (ROS) and other free radicals. These highly reactive species can be produced by enzymatic and non-enzymatic processes, can be endogenous or exogenous, and some of the commonly reported in cells are the hydroxyl ($\cdot\text{OH}$), superoxide ($\text{O}_2^{\cdot-}$), nitric oxide (NO), nitrogen dioxide ($\cdot\text{NO}_2$), peroxy ($\text{ROO}\cdot$) and lipid peroxy ($\text{LOO}\cdot$) radicals; other molecules, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1\text{O}_2$), hypochlorous acid (HOCl), peroxynitrite (ONOO^-) and lipid peroxide (LOOH), can easily lead to free radical reactions (157). Apart from their physiological role, their exacerbation is linked to various deleterious effects that include structural damage to DNA and membranes, changing the structure, alteration of the function of proteins or the induction of lipid peroxidation, that can ultimately lead to cell death (157).

The exacerbation of oxidative stress is a common feature of neurodegenerative disorders, and its toxic effects are manifested through different pathways (158). Neuronal cells are highly sensitive to oxidative stress because there is a relatively increased rate of oxidative metabolic activity, increased concentration of metal ions with age, rich content in polyunsaturated fatty acids prone to oxidation, and poor capacity to cope with oxidative stress (low levels of antioxidants and protective enzyme activity); additionally, once damaged, the non-replicating neuronal cells may be dysfunctional for life (159,160).

Flavonoids exert their protective effects against the exacerbated oxidative environment through multiple intertwined mechanisms (e.g., scavenging of radical species, chelation of

transition metals, avoiding the formation of hydroperoxides, or by enhancing the expression/activity of enzymatic antioxidant defence mechanisms), which are frequently designated as antioxidant cooperative actions (161).

Flavonoids directly scavenge free radicals; due to their lower redox potential, flavonoids are thermodynamically able to reduce by hydrogen atom donation the highly oxidizing free radicals with redox potentials in the range of 2.13–1.0 V (e.g., $\cdot\text{OH}$, $\text{O}_2\cdot^-$ and $\text{LOO}\cdot$) (162). From this reaction results a more stable and less-reactive radical, and a phenoxyl radical (flavonoid- $\text{O}\cdot$) that may further react with a second radical and acquire a stable quinone structure. In general terms, the B ring hydroxyl configuration is the most significant determinant of ROS scavenging, the polymerization of flavonoid monomers adds additional OH groups and increases the antiradical activity, and the glycosylation of flavonoids reduces their effect when compared to that of the corresponding aglycones. More specifically, the main structural features of flavonoids required for efficient radical scavenging are the presence of a catechol group (*O*-dihydroxyl structure) (**Figure 7A**) or of a pyrogallol group (hydroxylation at 3', 4'- and 5'- positions) in the B-ring (**Figure 7B**); a 2,3-double bond in conjugation with a 4-oxo function in the C-ring (**Figure 7C**); hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the oxo group (**Figure 7D**); the *C*-glycosylation in A-ring increases the antioxidant potential (**Figure 7E**) (163,164).

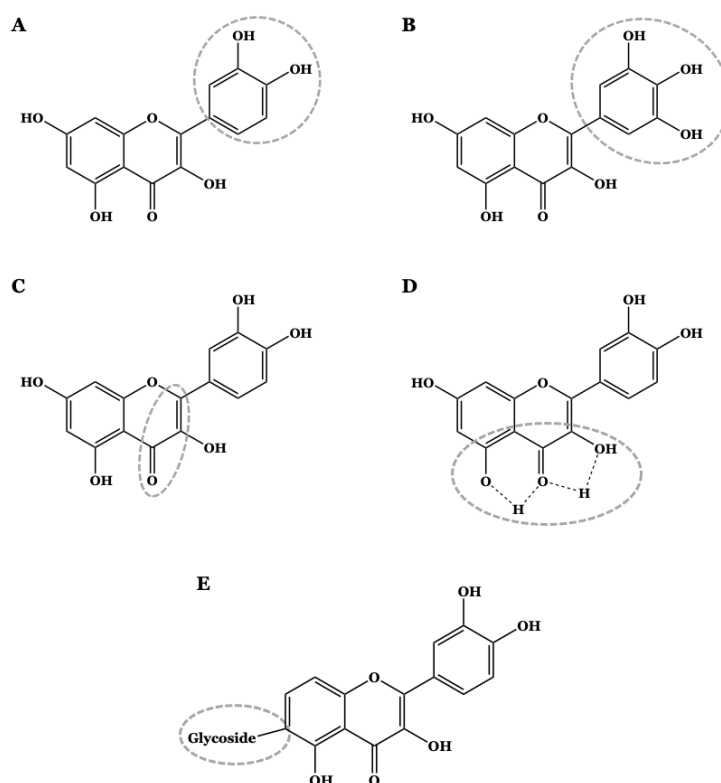


Figure 7. Structural features of flavonoids required to an efficient *in vitro* scavenging of reactive species.

Rather than ROS scavenging, there is the hypothesis that the inhibition of their generation may be a more successful strategy. There are several significant enzymatic and non-enzymatic endogenous sources of ROS. Metallochemical reactions are a common denominator since there seems to be a seminal relationship between redox-active metals, such as iron and copper, with the generation of oxygen and protein radicals that mediate the major neurological disorders (165). These metals play an important role in the generation of the very reactive and damaging hydroxyl radical through Fenton and Haber-Weiss reactions (166). Therefore, in addition to the relevant scavenging activity of this radical by flavonoids (167), it must also be considered their ability to strongly chelate prooxidant metal ions, thus removing a causal factor for the development of free radicals (168). In terms of structural features, it is proposed that the binding sites for metals on flavonoids are at α -hydroxy-carbonyl, β -hydroxy-carbonyl, and the catechol group in the B-ring (**Figure 8**) (169). Some authors also propose that the metal chelating activity of flavonoids can prevent metal-engendered oxidative stress and aggregation of proteins. In some neurodegenerative disorders was observed that protein aggregation in neuronal tissue is followed by cell site oxidation mediated by redox-active metal ion interaction with a target protein, leading to functional demise (165).

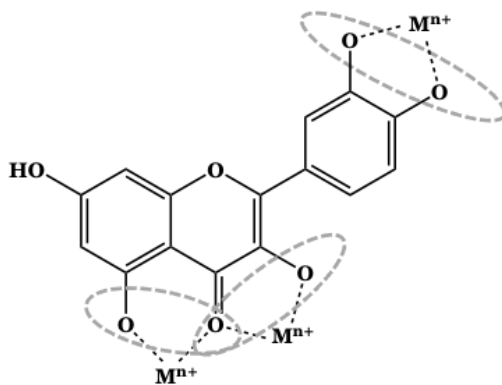


Figure 8. Binding sites for trace metals in the structures of flavonoids.

The mitochondrial ROS overproduction and changes in mitochondrial redox homeostasis have been shown to be involved in neurodegenerative processes (170). In mitochondria, the production of adenosine triphosphate is coupled to the flow of electrons down the electron transport chain, although *en route* to water some of the substrate derived electrons are diverted from the flow and participate in a single-electron reduction of oxygen, thereby converting it into superoxide (171). As discussed before, flavonoids can directly scavenge this radical. The superoxide radical alone exerts deleterious effects, but in addition it can undergo dismutation *via* superoxide dismutase (SOD) to form oxygen and hydrogen

peroxide (172), and the last can be further converted to highly reactive hydroxyl free radicals *via* Fenton reaction and further impair cellular function. Furthermore, some flavonoids can suppress ROS production by stabilizing mitochondrial respiratory chain complexes I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome c oxidoreductase) (173), which have the highest ROS-producing capacity (171). In fact, quercetin can concentrate in mitochondria and behave as a 'coenzyme Q-mimetic molecule' allowing normal electron flow along the entire electron transport chain in the presence of rotenone (174).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is another major generator of ROS. The discovery of phagocyte- and non-phagocyte NADPH oxidases (NOX) in astroglia and neurons reinforced the contribution of these enzymes to the pathology of chronic neurodegenerative disorders. For instance, the activation of NOX2 in microglia has been demonstrated in brains of AD patients along with upregulation of NOX1 and NOX3 in early-stage post-mortem AD brains (175,176), and it is up-regulated in the substantia nigra pars compacta of human PD (177). Therefore, a proper modulation of their activity might hold therapeutic value (178). These enzymes transport electrons across biological membranes and, in general, the electron acceptor is oxygen, and the product of the electron transfer reaction is superoxide (179). So far, it was demonstrated that flavonoids containing an unsubstituted catechol B-ring (quercetin, epicatechin, catechin, luteolin, and fisetin) did not inhibit endothelial NADPH oxidase activity in intact human umbilical vein endothelial cells, while mono-*O*-methylated and glucuronidated metabolites of these flavonoids were active within a micromolar range of concentrations (180).

Other contribution to the imbalance of oxidative stress is the monoamine oxidase (MAO) activity. The deamination of biogenic amines (e.g., dopamine (DA) and serotonin (5-HT)) results in the generation of hydrogen peroxide, which, by the subsequent activity of glutathione peroxidase (GPx) and glutathione reductase (GR), may additionally lead to the depletion of the endogenous pool of reduced glutathione (GSH) and increased calcium influx (181).

The role of xanthine oxidase (XO) in AD is supported by the effects of amyloid-beta peptides on hydrogen peroxide-metabolizing enzymes (182), and in PD by the suppression of hydroxyl radical generation mediated by the XO inhibitor allopurinol in animal models (183). This enzyme uses xanthine and hypoxanthine as reducing substrates and yields both superoxide and hydrogen peroxide. Its activity is not only elevated by mitochondria and NADPH derived ROS, as the depletion of intracellular ATP resulting from impaired mitochondrial respiration leads to conversion of adenine nucleotides to hypoxanthine and

xanthine, which are substrates for XO (161). *In vitro* structure-activity analysis indicated that the planar structure and the C2-C3 double bond increased both the affinity and inhibition; B-ring hydroxylation and the substitution at C3 were unfavourable for XO inhibition more profoundly than to their affinity; methylation greatly reduced the inhibition without affecting the affinity; the glycosidic substitutions decreased the inhibition and lowered the affinity to different degrees depending on the location (184).

Nonenzymatic lipid peroxidation plays a role in the pathogenesis of several neurodegenerative disorders, including AD (185), PD (186) and HD (187). The human brain is rich in polyunsaturated fatty acids (PUFA) that combined with increased rates of oxidative metabolic activity sets a scenario prone to increased lipid peroxidation, resulting in a variety of highly reactive electrophilic aldehydes with damaging effects (188). Briefly, in the initiation stage ROS attack an allylic carbon to form a carbon-centred radical, then this radical reacts with O₂ to produce peroxy radicals (LOO•), which can react with adjacent lipids to form a lipid hydroperoxide, thus repeating the cycle (**Figure 9**). The lipid hydroperoxides can decompose to produce multiple lipid peroxidation products that are biomarkers of the oxidative process (e.g., malondialdehyde (MDA), 4-hydroxynonenal, isoprostanes and neuroprostanes). Lipid peroxidation has also been reported in phospholipids, specifically in the form of oxidized phosphatidylcholine (PC) (189). Flavonoids can terminate this chain reaction by donating a hydrogen atom to the lipid peroxy radical (**Figure 9**). The *in vitro* structure-activity analysis revealed that in flavonoids the polyhydroxylated substitutions, a 2,3-double bond, a free 3-hydroxyl substitution, and a 4-keto moiety confer a more potent effect (190).

The interaction with intracellular endogenous antioxidants is another cooperative mechanism by which flavonoids can protect cells from increased ROS production. They not only prevent depletion of antioxidants by the direct ROS scavenging, as they up-regulate the production of the antioxidant enzymes SOD, GPx, GR and catalase, and elevate the levels of glutathione (GSH) (191). Furthermore, flavonoids can directly activate intracellular enzymes and increase their antioxidant activity, as observed for the interaction of quercetin and catechin with GPx, and for the interaction of rutin, quercitrin, myricetin and kaempferol with catalase (191).

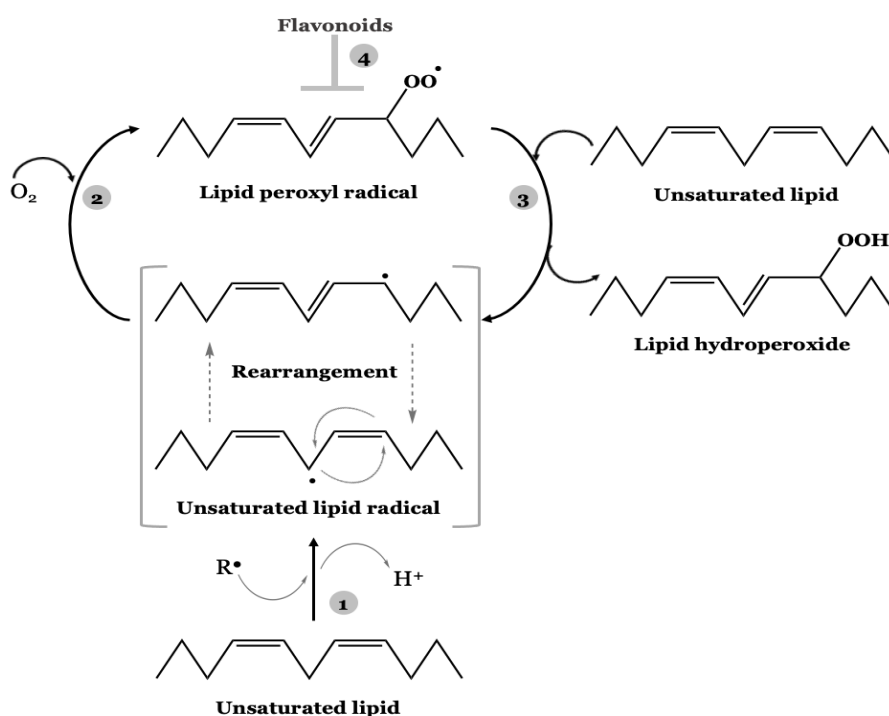


Figure 9. Nonenzymatic lipid peroxidation process. **1)** prooxidants, such as reactive oxygen species, abstract the allylic hydrogen thus forming a carbon-centred lipid radical, which tends to be stabilized by molecular rearrangement into conjugated diene; **2)** in the propagation phase, the lipid radical reacts with oxygen to form a peroxy radical; **3)** in its turn, the peroxy radical can abstract an hydrogen from another lipid, generating a chain reaction; **4)** flavonoids can donate an hydrogen to the lipid peroxy radical and break the chain process of lipid peroxidation (adapted from (191)).

1.2.3.3. Flavonoids regulate mitochondrial function

Many neurodegenerative diseases demonstrate abnormal mitochondrial morphology and biochemical dysfunction, but if mitochondrial dysfunction is a necessary step in neurodegeneration is still unclear (192). In general terms, mitochondria are key regulators of cell survival and death, and a dysfunction leads to reduced ATP production, impaired calcium buffering and increased generation of ROS that further accelerates the high mutation rate of mitochondrial DNA (mtDNA), all working in vicious cycles (193). As previously discussed, mitochondria-ROS generation has extremely deleterious effects. In addition, the conditions of excessive oxidative stress and mitochondrial calcium overload can activate the mitochondrial permeability transition (MPT) pore, resulting in the release of cytochrome c and the induction of caspase-mediated apoptosis. Furthermore, mtDNA is particularly susceptible to oxidative damage due to its vicinity with ROS sources and because it is not protected by histones, being hypothesized that somatic mtDNA mutations acquired during ageing contribute to the physiological decline that occurs with ageing and ageing-related neurodegeneration (193).

There is substantial evidence of morphological, biochemical, and molecular abnormalities in mitochondria in neurodegenerative disorders. In AD, some examples are the presence of amyloid precursor proteins (APP) and A β in mitochondrial membranes and in the matrix of neurons of AD brains, the increased production of free radicals by A β induction, decreased cytochrome oxidase activity, and inhibition of ATP production (194). In PD, both wild-type and mutant α -synuclein are found in the mitochondrial membranes and are determined to cause mitochondrial dysfunction, parkinsonism having been associated with several mtDNA mutations (195).

A large body of *in vitro* and *in vivo* data support that flavonoids affect mitochondria function through diverse mechanisms, such as modulation of antioxidant response mediated by the antioxidant enzymes, and ROS generation by complex I, complex III, and MAO, modulation of pathways engaged in mitochondrial biogenesis, regulation of the mitochondrial membrane potential, and by preventing mitochondrial-triggered cell death by changing the levels of pro- and anti-apoptotic proteins (173).

1.2.3.4. Flavonoids modulate the monoaminergic system

Monoamine neurotransmitters (5-HT, and the catecholamines DA and noradrenaline (NA)) are essential for signalling in the CNS and peripheral nervous system since they are involved in the regulation of movement, basal muscle tone, activity levels, mood, attention, sleep, and pain modulation, among many other vital functions (196). Therefore, alterations of monoamine synthesis, metabolism or transport result in impaired biogenic amine homeostasis and inherent disorders, including depression and neurodegenerative disorders (197). Pre-clinical data support the involvement of the modulation of monoaminergic system by flavonoids mediated by multiple and overlapping pathways (198–204).

MAO catalyses the oxidative deamination of monoamine neurotransmitters, thereby regulating their levels and biological functions, such as mood, emotion, motor, perceptual and cognitive functions. These enzymes are localized on the mitochondrial outer membrane, 5-HT and NA being more efficiently oxidized by MAO-A, while dopamine is primarily metabolized by MAO-B (205). MAO-B accounts for more than 80% of total MAO activity in the human brain (205).

In neurodegenerative disorders, including PD and AD, MAO-B has been proposed to play a primary role, though generating ROS in oxidation of monoamine substrates, in particular of dopamine, and ROS overflow further contributes to the oxidative synthesis of

neurotoxins, such as 6-hydroxydopamine (6-OHDA); additionally, MAO-B inhibition prevents the production of the neurotoxin 1-methyl-4-phenylpyrimidium ion (MPP⁺) from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (205,206). Despite that, levodopa DA substitution therapy has remained the mainstay pharmacological treatment for PD, interest has also focused on MAO-B as a secondary drug target. In fact, selegiline and rasagiline are MAO-B inhibitors used in clinic to provide symptomatic benefit in PD, because of the elevation of brain dopamine by inhibiting its breakdown, and more speculatively because MAO-B inhibition might prevent formation of damaging dopamine-derived oxidation products (207).

According to the “monoaminergic theory” of depression, the etiological origin of depression is a deficit in monoaminergic neurotransmission (noradrenergic, dopaminergic, and serotonergic) (208). Although MAO-A inhibitors are mainly used for mood disorders, MAO-A inhibition might also be neuroprotective. It seems to be associated with the prevention of neuronal loss through activation of signalling in mitochondria, since it mediates the increased expression of genes for anti-apoptotic, pro-survival and neurotrophic factors (205,209). Furthermore, depression may be an early manifestation of AD before cognitive symptoms become apparent (210), since soluble A β species have been shown to induce a depressive-like phenotype in AD animal models and it has been reported that in depressed patients A β peptide levels are increased (211).

In vitro studies demonstrate that flavonoids are MAO inhibitors. In general, the increasing number of hydroxyl groups on the scaffold decreases the inhibitory activity; the introduction of a sugar or glucuronic acid at C7, as well as the hydroxylation at C3, lowers the inhibition; MAO inhibition is strongly dependent on the presence of a catechol group and the unsaturation at the C2–C3 positions (212,213).

The precursor of DA, levodopa (L-DOPA), is commonly used to reduce PD symptoms. It is a substrate for DOPA decarboxylase and COMT. Peripheral DOPA decarboxylase is inhibited by drugs administered during PD treatment, but it is important to suppress the conversion of L-DOPA into methylated metabolites by COMT (214). *In vitro* and *in vivo* data indicate that flavan-3-ols are strong COMT inhibitors (215).

Furthermore, it has been documented that some flavonoids can also interact or increase the expression of several of these neurotransmitter receptors (198).

Recently, it was demonstrated *in vivo* that the progressive intracellular neuromelanin build-up to a certain threshold may trigger PD-like neuronal dysfunction, and neurodegeneration (216). Increasing evidence indicates that neuromelanin possesses

antioxidant and free radical scavenging properties, and its synthesis may represent an antioxidant mechanism to remove excessive cytosolic DA-derived quinones and semiquinones, thereby blocking their potential toxicity (216). However, it is hypothesized that in the catecholamine-producing regions of the brain the age-dependent accumulation of neuromelanin, which is generally regarded as resulting from non-enzymatic DA autooxidation, courses with the overexpression of brain tyrosinase. As so, a prominent brain tyrosinase activity with consequent neuromelanin synthesis is no longer regarded as a neuroprotective antioxidant mechanism through clearance of potentially toxic oxidized dopamine species, but as process leading to long-term accumulation of neuromelanin with deleterious consequences (216). Melanins are brown-black pigmented biopolymers (eumelanin, pheomelanin and neuromelanin) that derive from a biosynthetic pathway initiated with the hydroxylation of L-tyrosine to L-DOPA, which then serves as a precursor to both melanins and catecholamines, acting along separate pathways (217). Although the synthesis of peripheral melanin pigments is relatively well understood, the mechanism leading to neuromelanogenesis is still a matter of speculation, and the tyrosinase-enzymatic driven biosynthetic pathway is still not consensual (217)

When considering any therapeutic strategy aimed at modulating neuromelanin levels, it must be by achieving a tight regulation of its levels above its protective threshold and below its pathogenic threshold (216). Some pre-clinical data demonstrate that some flavonoids may have the potential to reduce tyrosinase mediated neurotoxic effects (218).

1.2.3.5. Flavonoids are cholinesterase inhibitors

The brain cholinergic system forms an extensive network of projection neurons, which innervate several brain areas. These neurons are defined as cholinergic because they contain the machinery for synthesizing and releasing acetylcholine for neurotransmission and are grouped in nuclei (219).

In the CNS, acetylcholine predominantly changes neuronal excitability, alters presynaptic release of neurotransmitters, coordinates the firing of groups of neurons, and it is involved in complex functions, ranging from movement to higher consciousness (220). The degeneration of brain cholinergic neurons associated with decreased levels of acetylcholine, choline acetyltransferase and acetylcholinesterase (AChE) have particular relevance in AD, but it is a common feature of all neurodegenerative disorders by affecting different nuclei (219). For instance, the profound degeneration of the forebrain cholinergic neurons is considered responsible for attention deficit and the impairment of information

acquisition and memory formation occurring in AD (221). The degeneration of the midpontine cholinergic neurons observed in PD strongly contributes to the postural and mobility deficits and to sleep behaviour disorder (222).

It is based on the cholinergic hypothesis of geriatric memory dysfunction that selective inhibition of AChE by donepezil or galantamine have been used for treatment of AD, to attenuate the cholinergic deficit (223). Remarkably, brain levels of another enzyme that hydrolyses cholinesterase, butyrylcholinesterase (BuChE), show progressive and significant increases in AD. Therefore, BuChE inhibition (tacrine and rivastigmine co-inhibit both AChE and BuChE) may result in additional specific input into frontal cortical structures, due to the more focused projections of BuChE-positive thalamic neurons to frontal brain areas and the predominant neuroanatomical localization of BuChE in the limbic system and subcortical regions (224).

AChE and BuChE share 65% amino-acid sequence homology, and both appear to be simultaneously active in the synaptic hydrolysis of acetylcholine to choline and acetate, thus terminating its neurotransmitter action and co-regulating levels of this neurotransmitter (224). BuChE plays a supportive role in the brain and accounts for about 10% cholinesterase activity in the temporal cortex (225). While AChE is highly selective for acetylcholine hydrolysis, BuChE can metabolize several different molecules including various neuroactive peptides. Furthermore, AChE has greater catalytic activity at low acetylcholine concentrations, but BuChE is more efficient at high substrate concentrations (224).

A large body of supporting *in vitro* data indicate that flavonoids, from all the different sub-classes, display efficient inhibition of either AChE or BuChE activities. From structural-activity relationship studies, it was concluded that the maximum AChE inhibitory activity displayed by flavonoids increases with the increasing number of free hydroxyl groups at A- and B-rings, when its present the C3–C4 double bound, and when gallate group is found (226). Some flavonoids exhibited a higher selectivity for binding to BuChE than to the related AChE (227).

Pre-clinical data also demonstrates that the effects of luteolin on learning acquisition in rats involves the central cholinergic system, by increasing the activities of central muscarinic and nicotinic receptors (228) and with increased levels of acetylcholine (229). Furthermore, luteolin promoted choline acetyltransferase activity and reduced AChE activity in the hippocampus of an AD model in rats (230). Similar findings were observed for other flavonoids and flavonoid-rich foods/extracts (231).

Overall, the outstanding preclinical efficacy of flavonoids as cholinesterase inhibitors suggests they may be good candidates for clinical trials that can lead to future clinical applications.

1.2.3.6. Flavonoids protect against glutamatergic dysfunction

Glutamate is the dominant excitatory neurotransmitter in human and plays a crucial role in the CNS, and its concentrations are tightly controlled by many mechanisms at the synapse because perturbations to this regulatory system can have deleterious effects (232). Glutamate receptors are numerous and highly complex, falling into two main categories; ionotropic (iGluR; NMDA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors) and metabotropic receptors (mGluR). iGluR are fast acting, and after glutamate binding, the receptor's channel undergoes a conformational change to allow an immediate influx of extracellular sodium, among other ions, and an efflux of potassium, thus triggering membrane depolarization in the post-synaptic cell to induce signal transmission (233). In addition to sodium and potassium ions, NMDA is permeable to calcium. mGluR are slower acting, and their activation results in diverse actions on neuronal excitability and synaptic transmission by modulation of a variety of ion channels, and other regulatory and signalling proteins (234). Group I receptors (mGluR1 and mGluR5) are normally stimulatory and associated with phospholipase C activation and second messengers, such as inositol and diacylglycerol production; Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7, and mGluR8) normally inhibit glutamatergic neurotransmission (234).

Glutamate transporters regulate glutamate concentrations and are situated on both pre- and post-synaptic neurons, as well as on glial cells. The excitatory amino acid transporters (EAATs) on glial cells (most often astrocytes, but also microglia and oligodendrocytes) perform a key role in modulating extracellular glutamate levels since it is recycled continuously between neurons and glia in what is known as the glutamate–glutamine cycle (235).

Glutamatergic dysfunction is a common pathway in several neurodegenerative disorders. Chronic glutamate-mediated neurotoxicity, also known as excitotoxicity, through overstimulation of iGluRs, such as NMDA receptor, but also by mGluRs, has been implicated in the disease onset and progression of several neurodegenerative diseases (236). In general, glutamate-induced neuronal damage involves an abnormal calcium influx mainly mediated by NMDA receptor that leads to the activation of calpains and other

proteases mediating cytoskeleton damage, paralleled by ROS generation, mitochondrial dysfunction, and subsequent neuronal apoptosis (237). As example, in the pathophysiology of AD, there is evidence for chronic excitotoxicity, which may be driven by multiple factors including the sensitization of NMDA receptors, a decrease in glutamate reuptake capacity, and an increase in glutamate release (238). Moreover, mGluRs have been proposed as promising therapeutic targets to correct the dysregulated glutamate signalling associated with neurodegenerative disorders (239). Of all mGluR subtypes, especially mGluR5 has been targeted for potential treatment of various neurodegenerative disorders and possesses some favourable characteristics. It acts as a modulator of glutamate-induced excitotoxicity; its modulation was shown to improve motor function in several movement disorders both clinically and in rodent models; it may act as a co-receptor to A β plaques and α -synuclein oligomers providing a direct link between mGluR5 and proteinopathies; the topographical organization of neuronal mGluR5 expression is restricted to cerebral regions affected by neurodegenerative diseases (240). The activation of mGluR7 lowers NMDA levels, thus it may protect neurons against such damage by diminishing excitotoxicity (241). Regarding glutamate transporters, alterations in EAATs expression and/or function have been increasingly suggested to take part in some neurological disorders. In some cases, there is convincing evidence that changes in EAATs precede neurodegeneration, whereas in other disorders alterations may result from neurodegeneration (242).

There is already sustained evidence that some flavonoids protect neurons against glutamatergic dysfunction. Apigenin is neuroprotective against glutamate-induced neurotoxicity in cerebellar and cortical neurons in culture, and the flavonoid was almost inactive on AMPA mediated currents while it reduced NMDA receptor mediated responses (243). In HT22 cells, the treatment with kaempferol resulted in a significant increase in cell viability, reduced glutamate-induced calcium influx, and reduced ROS generation (244). In primary neuronal cultures obtained from fetal rat cerebral cortex, myricetin modulated NMDA causing a subsequent reduction in glutamate-induced intracellular calcium overload and inhibited the ROS production (245). In both *in vitro* and *in vivo* models of ischemia, morin protected neurons from glutamate excitotoxicity by attenuation of receptor-mediated calcium influx, oxidative stress, and apoptosis (246). Luteolin protected rat brains from kainic acid-induced excitotoxic damage by reducing glutamate levels, mitigating inflammation, and enhancing Akt activation in the hippocampus (247). Hesperidin inhibits evoked glutamate release *in vitro* and attenuates *in vivo* kainic acid-induced neuronal death in the hippocampus by chelation of the extracellular calcium ions (248). Baicalein facilitates a postsynaptic NMDA receptor-dependent long-term potentiation (249), significantly attenuated muscle tremor of PD rats (250), attenuated the upregulation of striatal basal glutamatergic strength by decreasing the presynaptic glutamate release and decreased the

upregulation of cytokines in the substantia nigra and striatum in the mice injected with MPTP (251).

1.2.3.7. Flavonoids protect against GABAergic dysfunction

Compared to the marked deficits seen in excitatory cholinergic and glutamatergic systems, much less is known about the role of gamma-hydroxybutyric acid (GABA) in neurodegenerative disorders. GABA is an amino acid that serves as the primary inhibitory neurotransmitter in the human CNS, it exerts its primary function in the synapse between neurons by binding to post-synaptic GABA receptors, which modulate ion channels, hyperpolarizing the cell and inhibiting the transmission of an action potential (252).

In GABAergic interneurons, neurotransmitter GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase, transported along the axon to presynaptic terminals and recruited into vesicles by the vesicular GABA transporter, and then it can be released from presynaptic vesicles into the synaptic cleft resulting in the burst increase of GABA concentration in the cleft. There are two main types of GABA receptors, the ionotropic GABA_A receptor and the metabotropic GABA_B receptor. When GABA activates GABA_A receptors the ion channel opens and chloride diffuses into the cell, thus hyperpolarizing the post-synaptic mature neuron. In contrast to the ionotropic GABA_A receptors, GABA_B receptors are found in pre and post synaptic neurons and are responsible for the later and slower component of inhibitory transmission *via* G-protein mediated pathway that activates various intracellular signal cascades, and ultimately either to activation of postsynaptic potassium channels or inhibition of presynaptic calcium channels (252).

The clinical significance of GABA cannot be underestimated, and there is increasing evidence that GABAergic neurotransmission also undergoes profound pathological changes in neurodegenerative disorders, thus a promising therapeutic target. For instance, in the temporal cortex of AD patients were observed significantly lower levels of GABA; the loss of GABAergic neuronal function in brain sections from AD patients, especially on cortical neurons adjacent to amyloid plaques; GABA_A receptor activation may enhance tau phosphorylation and consequently increase intracellular neurofibrillary tangles in neurons and contribute to the development of AD; vice versa, hyperphosphorylated tau may enhance GABAergic neurotransmission (253). There is also increasing evidence of abnormal GABAergic neurotransmission in PD (254).

There has been a resurgence of interest in flavonoids as modulators of GABA_A receptor. Flavonoids are ligands for benzodiazepine binding sites on GABA_A receptors and exert a benzodiazepine-like anxiolytic effect (200,255,256), but it has become apparent that this interaction is far more complex than a single action at this one site. In fact, new topics are being explored, such as if flavonoids that show subtype selectivity in recombinant receptor studies *in vitro* are consistent with their behavioural effects *in vivo*, or the identification of the different sites of action of flavonoids on GABA_A receptor complexes (257).

Single channel studies may shed light on the different mechanisms of action of flavonoids on GABA_A receptors.

- Identification of the various flavonoid binding sites and their possible overlap with binding sites for other agents acting on GABA_A receptors, for example etomidate and loreclezole.
- Flavonoids that bind to GABA_A receptors but show no intrinsic activity in functional studies should be investigated as possible antagonists.

As significant constituents of our diet, it is important that we understand how natural flavonoids might influence brain function. Understanding the structural determinants of flavonoid effects on GABA_A receptors and development of agents specific for GABA_A receptor subtypes is a key challenge. Synthetic flavonoids are attractive leads not only for drugs to treat brain dysfunction, but also for the development of molecules that can be used to study and investigate the role of the modulatory sites at GABA_A receptors and further development of GABA_A subtype-selective agents.

1.2.3.8. Flavonoids in the modulation of neuroinflammation

The neuroinflammatory process is a response that involves all the cells present within the CNS, including neurons, macroglia and microglia, with beneficial effects by promoting tissue repair, removing cellular debris and pathogens (258). This process is mediated by the production of cytokines, ROS and secondary messengers that are mainly produced by resident CNS glia (microglia and astrocytes), endothelial cells and peripherally derived immune cells, and the extent depends on the context, duration, and course of the primary stimulus or insult (259).

Microglia and astrocytes are key regulators of inflammatory responses in the CNS. However, it is established that a dynamic crosstalk exists between BBB endothelial cells, glia, and neurons, and it is likely that a neuro-inflammatory response from one type of cell

will directly impact another (260). Microglia and astrocytes serve diverse functions including innate immune responses in the brain and are traditionally classified into two opposing phenotypes: neurotoxic and neuroprotective. Microglia are divided into M1 (classical activation) and M2 (alternative activation) phenotypes based on their activation status, likewise astrocytes that can produce pro-inflammatory or immunoregulatory mediators according to the phenotype of the polarization status (261). Microglia are ubiquitously distributed in the brain and are the principal innate immune cells, by detecting changes in their environment using their sensomes, physiological housekeeping function, including migrating to injured sites, remodelling synapses, and maintaining myelin homeostasis, and by protecting against injurious stimuli mediated by cellular receptors, such as toll-like receptors (TLRs), nuclear oligomerization domain-like receptors, and viral receptors (261). In response to such stimuli, microglia produce NO, proinflammatory cytokines (e.g., tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-16) and chemokines (e.g., the C-C motif chemokine ligand 2 (CCL2) and IL-18). In contrast, IL-4, IL-10, IL-13, and transforming growth factor- β (TGF- β) activate neuroprotective microglia (261). Astrocytes play important physiological roles (e.g., regulate blood flow, maintain the BBB, provide metabolites to neurons, modulate synaptic activity, control neurotrophin secretion and remove dead cells), and, similarly to microglia, they have pro-inflammatory subpopulations that upregulate several genes (e.g., complement cascade genes) and induce pro-inflammatory factors (e.g., IL-1 β , TNF- α , and NO), and immunoregulatory subpopulations that release anti-inflammatory cytokines (e.g., IL-4, IL-10, and TGF- β). Furthermore, they upregulate the transmembrane receptors for IL-17 and tropomyosin receptor kinase B (TrkB) during neuroinflammation, resulting in the recruitment of nuclear factor κ B (NF κ B) activator and production of pro-inflammatory cytokines, and express signal transducer and activator of transcription 3 (STAT3) after injury (261).

In terms of the molecular components of neuroinflammation, cytokines are cell-signalling proteins that mediate neuroinflammation, causing its exacerbation or reduction. Pro-inflammatory cytokines such as TNF- α and IL-1 β play an integral role in pathological inflammation and acceleration of disease, while IL-4 is largely anti-inflammatory. On binding to the extracellular TNF receptor-1, TNF- α sets off several signalling cascades that affect gene transcription, for instance, the activation of NF- κ B and induction JNK/SAPK pathways, which activate various other transcription factors that modulate apoptosis and inflammation (260). When bound to the IL-1 receptor complex, IL-1 β initiates the other MAPK, ERK and p38 MAPK, which activation results in many pro-inflammatory responses (260). Chemokines are involved in the upregulation and chemotaxis of glial cells, and they may also disrupt neuronal function and adversely affect neurogenesis. The complement cascade is an important feature of immunity and inflammation because it contributes to

processes like mast cell degranulation, chemotaxis, and cell lysis (260). Chronic inflammatory responses, however, are detrimental, specially along with the persistent inflammatory responses of microglia and astrocytes, which and can lead to neurodegenerative disorders.

NO is a pleiotropic molecule synthesized by nitric oxide synthases (NOS) through the conversion of L-arginine to NO and L-citrulline, which plays a critical role in a few physiological and pathological processes. Three isoforms of NOS have been identified in CNS: NOS1, or neuronal NOS (nNOS), localized at the synaptic spines, astrocytes and the loose connective tissue surrounding blood vessels in the brain; NOS2, or inducible NOS (iNOS), a Ca^{2+} -independent isoform not constitutively expressed by astrocytes and microglia, but often expressed in response to inflammatory stimuli; NOS3, or endothelial NOS (eNOS), present in both cerebral vascular endothelial cells and in motor neurons (262). Excessive NO production, as the evoked by inflammatory signals, has been identified as one of the major causative reasons for the pathogenesis of several neurodegenerative disorders. There is an intimate relation between microglial activation, NO and neuroinflammation in the human brain. NO can induce oxidative and nitrosamine stress, which activates mitochondrial apoptosis by several pathways, including the stimulation of mitochondrial permeability transition, up-regulation of pro-apoptotic genes, activation of the p38 MAPK pathway, and induction of endoplasmic reticulum stress; it can modulate the inflammatory process acting on key regulatory pathways, such as those associated with excitotoxicity processes induced by glutamate accumulation and microglial activation; activated glia express iNOS and produce NO that triggers calcium mobilization from the endoplasmic reticulum, activating the release of vesicular glutamate from astroglia cells, resulting in neuronal death (262).

The lipid metabolism is also involved in the neuroinflammatory process. PUFA metabolism is mediated by lipoxygenases (LOXs) that catalyse the addition of oxygen, leading to the formation of bioactive lipids. Among the several isoforms, 5-LOX and 12/15-LOX isoforms are especially important in neuroinflammation/neurodegeneration, since they are up-regulated by signalling pathways, such as MAPK (263). The enzyme cyclooxygenase (COX) converts arachidonic acid to eicosanoid groups, such as prostaglandins and thromboxanes, and the pathways of its two common isoforms COX-1 and COX-2 are becoming increasingly associated with neuroinflammation and neurodegeneration, COX-1 expression leading to prostaglandin synthesis in microglia, while COX-2 expression is mainly observed in neurons and is associated with synaptic functioning and memory formation (260).

Microglia response leads to increased levels of pro-inflammatory mediators (e.g., IL-1 β , TNF- α , ROS and NO) that at a basal level would act as scavengers to deal with normal turnover of neurons and help clear the debris of necrotic neurons. However, in neurodegenerative diseases a chronic and exacerbated microglia response may aggravate the condition by releasing too much of these cytotoxic factors and resulting in progressive neuronal cell death (264). For instance, in AD, the A β deposits, instead of being efficiently removed by microglia, can trigger these cells to secrete proinflammatory cytokines, further damaging neurons. In PD, α -synuclein can also be nitrated by the NO produced from activated microglia, and α -synuclein thus modified post-translationally can promote the death of dopaminergic neurons (265).

Once neuroinflammation is deeply intertwined in neurodegenerative disorders, the modulation of engaged effectors or targets may be useful to prevent or delay harmful effects. Flavonoids and flavonoid-rich extracts modulate the inflammatory responses involved in neurodegenerative diseases primarily related to the regulation of microglial cells mediated by their effects on MAPKs and NF- κ B signalling pathways (e.g., reduction of the expression of the pro-inflammatory cytokines TNF- α , IL-1 β , down-regulation of inflammatory TLR-4 and COX-2 signalling cascades, or modulation of NADPH oxidases-ROS pathways), as demonstrated by *in vivo* and *in vitro* data (266).

1.2.3.9. The multifunctional role of flavonoids against the formation of AGEs and their induced harmful effects

Advanced glycation end-products (AGEs) are a group of cross-linked irreversible adducts created by non-enzymatic glycation of free amino groups of proteins, lipids, or nucleic acids with reducing sugars. In the first step of protein-AGE formation, a non-enzymatic reaction (Maillard reaction) occurs between a free amino group and the carbonyl group of a reducing sugar (e.g., glucose). This step is followed by a reversible nucleophilic addition reaction that results in a Schiff base, which slow chemical rearrangements, over a period of weeks, lead to the stable Amadori product that interacts again with free amino groups of proteins, thus amplifying the Maillard reaction. Finally, Amadori products undergo rearrangements, dehydration, and rearrangement, with/without oxidation, to form dicarbonyl precursors (e.g., glyoxal and methylglyoxal) that cross-link with adjacent proteins AGEs (267). Notably, the latter reactions of the adducts can be accelerated by transition metals, which oxidize the protein-bound Amadori products (268). The formation of AGEs is irreversible and leads to the deposition of proteins due to the protease resistant crosslinking between the AGE-containing peptide fragments and proteins. Alternative

pathways of AGE formation include the carbonyl stress pathway, where oxidation of sugars and/or lipids generates dicarbonyl precursors (e.g., glyoxal and methylglyoxal) to form AGEs. Another alternative is the formation of AGEs through polyol pathway (267).

The receptor for AGEs (RAGE) is a multiligand cell surface receptor expressed in various cells, including endothelial cells, macrophages, microglia, astrocytes, and neurons (269), which is upregulated in aging-related pathologies. The interaction of AGE with RAGE triggers intracellular and extracellular signalling pathways, thus increasing oxidative stress and inflammation that, in turn, promotes RAGE expression in a positive feed-forward loop contributing to chronic disease development (270).

As already discussed, neurodegenerative disorders like AD and PD are characterized by the abnormal accumulation or aggregation of proteins. AGEs contribute to the development of neurodegenerative disorders because glycation alters the biological activity of proteins and their degradation processes, protein cross-linking by AGE results in the formation of detergent-insoluble and protease-resistant aggregates, and glycation reactions lead to the production of reactive oxygen species (271). More specifically, AGEs accumulation has been detected in different cortical areas of AD brain, the polymerization of A β was shown to be significantly accelerated by AGEs mediated protein cross-linking, amyloid precursor protein expression was up-regulated by AGEs *in vitro* and *in vivo* leading to increased A β -levels; furthermore, AGEs contribute to the formation and toxicity of neurofibrillary tangles, which are primary markers of AD (268). In the pathogenesis of PD, histochemical analysis demonstrated that AGEs and RAGE levels were increased in the frontal cortex of PD patients compared to controls (268).

Flavonoids inhibit the *in vitro* formation of AGEs when experiments are conducted in chemical models (e.g., glycation of bovine serum albumin by glucose, methylglyoxal, or fructose), cell models (e.g., glycation induced in human monocytic THP-1 cells by glucose), or even animal organs (e.g., bovine lens organ or goat lens-fructose cultures) (272). In these models, flavonoids can prevent AGE formation *via* antiradical and metal ion chelation activities (273), or by trapping dicarbonyl precursors (274). The antiglycative ability of flavonoids in animal studies was reported for fisetin, catechins and phloretin (272). Furthermore, kaempferol (a flavonol) revealed to attenuate AGEs-induced damage possibly by targeting RAGE, thus, through modulation of signalling pathways (275).

Other relevant role of flavonoids is in the enhancement of the glyoxalase pathway (276). This pathway is a crucial antioxidant system for defence against reactive dicarbonyl compounds like the methylglyoxal detoxification into D-lactate, which can then be converted into pyruvate, the main substrate used to generate ATP through aerobic

respiration. The treatment of cerebellar neurons prepared from mice with catechin, morin, and quercetin protected neurons from methylglyoxal-mediated damage by multiple mechanisms, as verified by the increased concentration of GSH (initially GSH reacts with methylglyoxal to form a hemithioacetal), the overexpression of glyoxalase enzymes (glo-1 and glo-2, that mediate methylglyoxal detoxification into D-lactate), and by the reduced expression of apoptotic proteins (277).

1.2.4. Flavonoids and nanotechnology: an opportunity to enhance their bioavailability and bioefficacy

1.2.4.1. CNS bioavailability of flavonoids

The effectiveness of flavonoids to manage neurodegenerative disorders will depend on their pharmacological concentrations within the CNS. As previously referred, the bioavailability of dietary flavonoids is conditioned by their physicochemical characteristics and the structural alterations endured following their absorption and metabolism.

When CNS is the target, flavonoids must cross another barrier, the blood-brain barrier (BBB). This physical interface ensures a proper CNS homeostasis due to a very selective and restrictive bidirectional transport of endogenous and exogenous compounds. Morphologically, the functional unit of the BBB comprehends the endothelial cells with the surrounding microglia, pericytes, astrocytes and neurons (**Figure 10**). This complex network features junctional complexes (tight, adherens and gap junctions) with almost complete absence of fenestrae, and the imparted high trans-endothelial electrical resistance that together strict the paracellular permeability between the endothelial cells (278). On the other hand, the transport *via* cytoplasm is strongly hindered by the transcellular barrier effect, a result from the low density of pinocytotic vesicles and low levels of endocytosis and transcytosis (278). Furthermore, other peculiar features like metabolic enzymes (e.g., β -glucuronidase and alkaline phosphatase (ALP)), membrane transporters and receptors are the final key to endure such a restrictive permeability (279).

The bioavailability of flavonoids will be dictated by the structural characteristics of each compound together with their interactions with the BBB elements. The experiments conducted with diverse *in vitro* cellular models of BBB demonstrate that, excepting quercetin, there is a significant relation between the *in vitro* calculated apparent permeability coefficient (P_{app}) of the flavonoids and their oil/water partition coefficients

(logP). Flavonoids with an increased number of hydroxyl groups and sugar moieties have higher logP values (quercetin-3-*O*-rutinoside > hesperetin-7-*O*-rutinoside > quercetin > kaempferol > apigenin > isoliquiritigenin), and an almost totally overlapping correspondent lower P_{app} value (quercetin < quercetin-3-*O*-rutinoside < hesperetin-7-*O*-rutinoside < kaempferol < apigenin < isoliquiritigenin) (280). In addition, the permeability of flavonoids seems to be facilitated when the hydroxyl groups in the B-ring are placed in *ortho*- rather than in *meta*-position (280). In relation to the aglycones, their glycosylation increases logP values and lowers the permeability, and this effect is even more pronounced for the more polar glucuronide derivatives (281). In contrast, since *O*-methylated flavonoids have higher lipophilicity, they also have a higher transport efficiency (280,282).

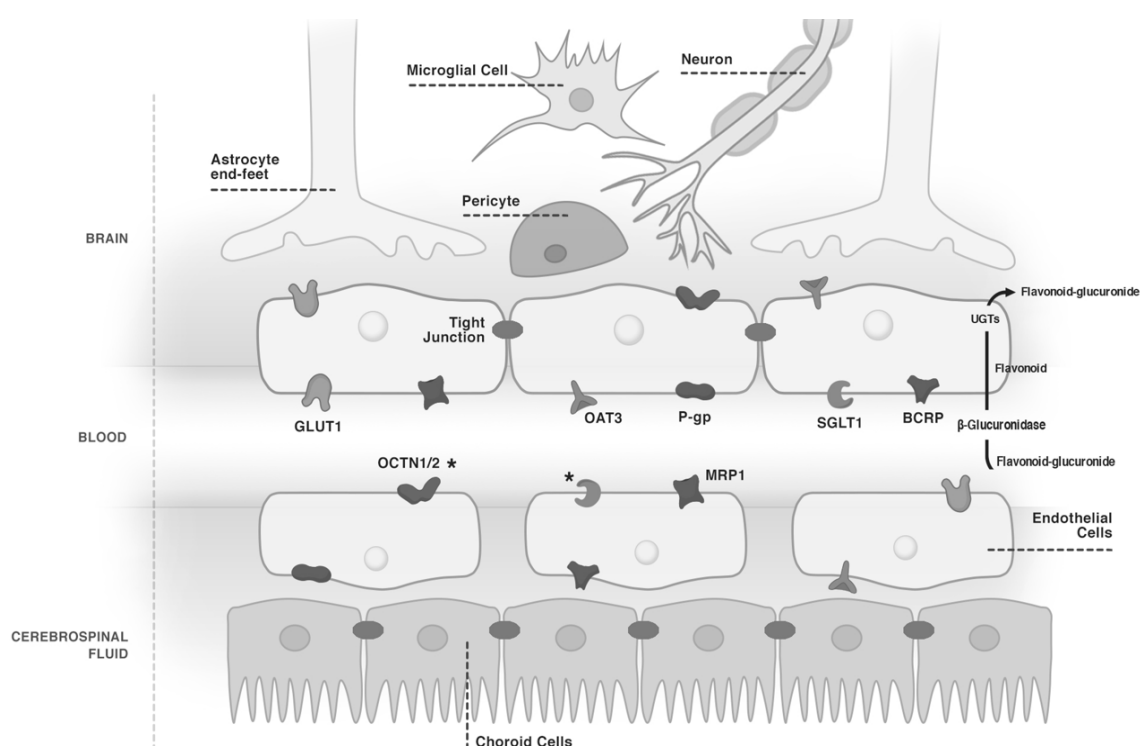


Figure 10. Illustration of the neurovascular unit of the blood-brain barrier and of the blood-cerebrospinal fluid barrier (adapted from (283)).

However, the logP alone is not enough to predict the *in vitro* membrane permeability for flavonoids. For instance, in several works conducted with diverse models, the calculated permeability for quercetin was consistently inferior to that of other flavonoids with lower lipophilicity (280,284,285).

In fact, flavonoids interact with membrane transporters that can either enhance (influx transporters) or impair (efflux transporters) their transport across the BBB (286). The expression and activity of drug transporters in the endothelial cells is a limiting factor

for either flavonoids or their metabolites, and it is mediated by the solute carriers (SLC) and ATP-binding cassette (ABC) transporters superfamilies. In general, the SLC superfamily (e.g., glucose transporter 1 (GLUT1/*SLC2A1*) and sodium-dependent glucose transporter 1 (SGLT1/*SLC5A1*)) is expressed in plasma membranes (**Figure 10**) and can take part in uptake mechanisms, and sometimes in efflux mechanisms (287). The multidrug resistance-related proteins (MRP) family (e.g., P-gp, breast cancer resistance protein (BCRP/*ABCG2*), and MRP1/*ABCC1*) is more related with extrusion at the luminal membranes (**Figure 10**) (288).

Flavonoids, quercetin in particular, are well-known substrates for P-gp, which can efflux them from the cells (284). They can also act as competitive P-gp inhibitors, and even bind at multiple sites and acting as bifunctional modulators (289,290). In addition, some flavonoids (quercetin and kaempferol) display a concentration-dependent biphasic effect over the uptake of other drugs (291,292). *In vitro* structure-activity analysis demonstrated that flavonols, chalcones and flavones are generally active inhibitors; the lowest inhibition is observed with flavanones, and no interaction exists with quercetin-3-*O*-rutinoside and other highly hydrophilic flavonoid glycosides; free hydroxyl groups at positions 3 and 5 are required for the inhibitory activity, and there is a correlation between increasing hydroxylation and increased affinity; the affinity towards P-gp increases with methoxylation at C4' and when the C2–C3 double bond is present, but decreases with *ortho*-hydroxylation (293). However, the cellular efflux of flavonoids may not be a one-transporter exclusive task, as observed with the transport of quercetin or naringenin mediated by both P-gp and BCRP (282,285). Still from this superfamily, the MRPs-mediated transport of flavonoids in the CNS is almost unexplored, although it was not observed any effect for quercetin, naringenin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, catechin or epicatechin (285,294). Within the SLC superfamily, the GLUT1 transporter seems to be involved in the passive transport of anthocyanins (295), and SGLT1 behind the dissimilar stereoisomeric transport efficiency of catechin and epicatechin (294).

Besides membrane transporters, the contribution of the mutual interactions between enzymes and flavonoids to their permeability cannot be depreciated (296). For instance, ALP activity is suggested as a regulatory mechanism in the transport of quercetin (282) and kaempferol (297). Other example is the transient deconjugation reaction mediated by β -glucuronidase, in the epithelial vicinity, that releases the aglycones and is followed by their intracellular re-conjugation mediated by UGTs (**Figure 10**). This phenomenon was described for quercetin-3-*O*-glucuronide (282,298), naringenin and hesperetin glucuronides (281), and some flavan-3-ols (294). The possible involvement of β -glucuronidase in the uptake of flavonoid glucuronyl conjugates not only reinforces the

contribution of endothelial cells to a controlled homeostasis, as it also suggests that glucuronides may serve as flavonoid carriers (299).

Despite the remarkable advances in the design and development of experimental models with more resemblances with the anatomic and physiologic structures, the linear translation of the *in vitro* findings to *in vivo* models is not always feasible (300). Free flavonoid aglycones are not usually detected in the blood stream, although diverse aglycones from the diverse sub-classes were found in rat brain tissues after dietary intake, oral or intraperitoneal administration (301–304). The uptake rate of quercetin is conditioned by its extrusion mediated by BCRP and P-gp transporters (285), its levels are lower in brain tissue than in others (lungs, white fat, spleen, liver, and heart) (305), but its dietary bioavailability increases when a quercetin glycoside is orally administered (306). Flavonoid glucuronides may serve as carriers, as these conjugates are found in rat brain tissues after oral administration of the parent compounds (306), thus suggesting the involvement of the transient deconjugation reaction mediated by β -glucuronidase. Anthocyanins are, by far, the most studied flavonoids in terms of pharmacokinetics. Their absorption is affected by food matrix effects, have a reduced half-life time, are detected in the CNS within 30 minutes after oral administration, and their phase II metabolites have a peculiar preferential brain tissue location (307).

1.2.4.2. Flavonoids in liposome drug-delivery systems

The therapeutic outcome of flavonoid-based therapies is dependent on the improvement of their pharmacokinetic profile. Flavonoids have poor water solubility, even if they are in the form of glycosides, and as previously referred, their bioavailability can be easily modified by matrix effect, pH variations, absorption, and metabolism, and by transporters and enzymatic activity. Notwithstanding, the adjustment of these variables may be regarded as potential strategies to overcome the bioavailability limitations, by increasing solubilization, altering absorption pathways or preventing the metabolic degradation.

Novel drug delivery systems (NDDS), such as those involving nanotechnology, constitute a novel approach in biomedical sciences, mainly owing to their small particle size, the capacity to transport molecules of distinct polarities, protection against metabolic pathways, opportunity for controlled or stimuli-responsive drug release, or to the possible enhancement of the targeted and intracellular delivery of drugs (308).

The development of nanoformulations to enhance the bioavailability and bioefficacy of single flavonoids includes inorganic nanoparticles, polymer-based carriers (nanocapsules, nanoparticles and polymeric micelles), and lipid-based carriers (liposomes, micelles, nanostructured lipid carriers, solid-lipid nanocapsules, and microemulsions) (309,310).

Lipids are at the headway of nanotechnology conveyance for the development of NDDS with noteworthy applications accomplished in the fields of cosmetic, functional foods and pharmaceutical formulations (311). In particular, phospholipids, are considered appealing structural platforms for lipid-based nanocarriers of bioactive compounds, because they have a self-assembling into amphiphilic structures, have an excellent biocompatibility, good emulsifying capacity, which can stabilize the emulsions, and as surface-active wetting agents they can enhance the hydrophilicity of hydrophobic drugs through surface coating of crystals (312). The basic structure of phospholipid consists of two hydrophobic fatty acid chains and a hydrophilic phosphate group, joined together by a glycerol molecule. The variation in head and tail groups, aliphatic chains and alcohols leads to the existence of a large range of phospholipids. The main natural sources for extracting phospholipids are vegetable oils (e.g., soybean and cotton seed) and animal tissues (e.g., egg yolk and bovine brain) (312).

When dispersed in water, phospholipids spontaneously self-assemble into closed spherical shaped structures with the internal aqueous environment bounded by phospholipidic bilayer membranes; this vesicular system is called liposomes (313). Liposomes can be formulated and processed to differ in size (e.g., small vesicles with diameter between 20–100 nm, and giant vesicles with diameters higher than 1 μm), composition (phospholipids, such as PC, are essential components, but cholesterol is frequently added), charge, and lamellarity (e.g., unilamellar and multilamellar vesicles) (314). The wide application of liposome-based drug delivery systems is based on some advantages, such as their biodegradability and biocompatibility characteristics, they can load both hydrophilic and hydrophobic drugs, their preparation is easy, and it is possible to modulate their pharmacokinetic properties by changing the chemical composition of the bilayer components (312,313). In addition, liposomal delivery systems are suitable agents to enhance the delivery of drugs into the CNS by different routes of administration (314,315).

When it comes to the loading of drugs in liposomal nanoformulations, flavonoids are no exception. These compounds, as other polyphenols, are neither considered fully lipophilic nor hydrophilic, but amphiphilic structures necessitating both hydrophobic and hydrogen bond interactions to be encapsulated. Although not fully understood, their interactions with

lipid bilayers include the partition of the more non-polar compounds in the hydrophobic bilayer of the membrane, and the formation of hydrogen bonds between the more hydrophilic polyphenol and the polar head groups of lipids and at the membrane interface (316).

In general, the neuroprotective effects of flavonoid liposomal nanoformulations observed in pre-clinical models hold a promising future therapeutic application. The bioavailability of quercetin, catechin and fisetin was improved when using liposome nanoformulations containing each aglycone (317). Quercetin liposomes protected against ischemic lesion through a significant increase in the number of cells in striatum and cortex, together with a partial reversal of motor deficits and increased GSH levels (318). Mannosylated quercetin liposomes preserved the activities of antioxidant enzymes and markedly inhibited cellular oedema formation in neuronal cells of young and old rats, in a cerebral ischemia-reperfusion-induced oxidative damage model (319). The intranasal administration of quercetin liposomes modulated cognitive impairment and inhibited AChE activity (320), displayed anxiolytic-like activity (321,322), attenuated the degeneration of cholinergic neurons, along with the elevation of antioxidant enzyme activity and reduction of lipid peroxidation (323). Luteolin liposomes acted as a neuroprotectant, likely through a rebalance of pro-oxidant/antioxidant status, thus resulting in anti-ischemic activity (324,325).

Lipid vesicles are versatile drug-delivery platforms that may also be carriers for plant extracts, denominated as phytosomes. In theory, these phyto-phospholipid supramolecular nanostructures feature more attractive pharmacokinetic and pharmacodynamic profiles than the conventional herbal preparations (326).

So far, data on either the bioavailability or neuroprotective effects of phytosomes is scarce. The aqueous extract of *Annona muricata* L. leaves, with total phenolic content composed by approximately 80% of flavonoids, was used to produce phytosomes functionalized with a modified peptide derived from the receptor-binding domain of apolipoprotein E (327). These phytosomes improved membrane permeability of the extract in an *in vitro* transwell model of BBB composed of immortalized human microvascular endothelial cells (hCMEC/D3). The administration of phytosomal curcumin led to a dose-dependent reduction in neuroinflammatory markers in the hippocampus and cerebellum of mice, thus attenuating the induced inflammatory condition and potentially reversing the detrimental effects of chronic glial activation (328). The administration of *Ginkgo biloba* L. phytosomes improved the pharmacokinetics of the herbal formulation (329). *G. biloba* phytosomes, prepared with a standardized extract with 20% of flavonoid content, increased

SOD, catalase, GPx and GR activities in all the brain regions of Wistar rats and prevented the depletion of the antioxidant enzymes by sodium nitrite (330). In another study with rodents, *G. biloba* phytosomes exhibited both anti-amnestic and antidepressant activities in the scopolamine-induced amnesia test and behavioural despair test, respectively (330). Phytosomes loading the combined extracts of *Morus alba* L. fruit and rhizomes of *Zingiber officinale* Roscoe (extracts with a total flavonoid content of ~90 g quercetin/mg of extract) protected rat brains against induced cerebral ischemia by increasing antioxidant enzymes and reducing inflammatory stress (331). The treatment with a phytosomal preparation containing *Centella asiatica* L. and *Curcuma longa* L. improved signalling pathways leading to reversion of impairments in neuroplastic mechanisms, memory, and cognitive abilities (332).

Flavonoids are present in some of these phytosome nanoformulations. However, despite their pleiotropic potential, the development of phytosomes with flavonoid-rich extracts and the evaluation of their neuroprotective effects is yet to be fully explored.

1.3. Selection of medicinal species

Among the plethora of medicinal species, in each study, it is important to define the premises that will guide the selection of the study material. Herein, the species were selected for their use in Traditional Medicine as neurostimulants, also because they are available to consumers in herbal stores, and there is a significant level of evidence that their biological activities may be related to the phenolic composition of their extracts.

1.3.1. *Trichilia catigua* A.Juss.

Trichilia catigua A.Juss. (Meliaceae) is a species widely distributed in South and Central America. It is a tree that grows up to 10 m high, with leaves composed by 5–7 leaflets that are short pedicellate, oblong-elliptic, acuminate leaf apex, acute at the base and have up to 7 cm in length. Its flowers may be whitish-yellow, and the fruit is a narrow oblong reddish capsule with long, stiff, yellowish hairs, and only one seed, appearing from December to January (333).

In Brazil, the stem bark of *T. catigua* (**Figure 11**) is known by “catuaba”. The macroscopy analysis shows a greyish outer surface, ranging from light to dark tones, with a

coarse granular appearance, small circular lenticels, and short and surface longitudinal cracks; the internal surface is reddish with finely striated fibres; its odour is not characteristic, and the taste is strong and bitter (334). The stem bark is the vegetable raw material of this species that is used in folk medicine for physical and mental fatigue, and as aphrodisiac (335).



Figure 11. *T. catigua* stem bark (photography by João Bernardo).

1.3.1.1. Phenolic composition of *T. catigua* stem bark extracts

In Brazilian folk medicine, the stem bark of *T. catigua* is used to prepare hydro-alcoholic decoctions designated by “Catuama” that have a rich phenolic composition. For analytical purposes, other type of extracting solvents, such as acetone, ethyl acetate, methanol, or even the quaternary water–methanol–acetone–ethanol mixture (1:1:1:1) are also used (336).

The phenolic compounds usually identified include phenolic acids (**Figure 12**) (337), flavonoids (**Figure 13**) (337,338), phenylpropanoid-substituted flavan-3-ols (**Figure 14**) (338–341), and procyanidins (**Figure 15**) (338,341).

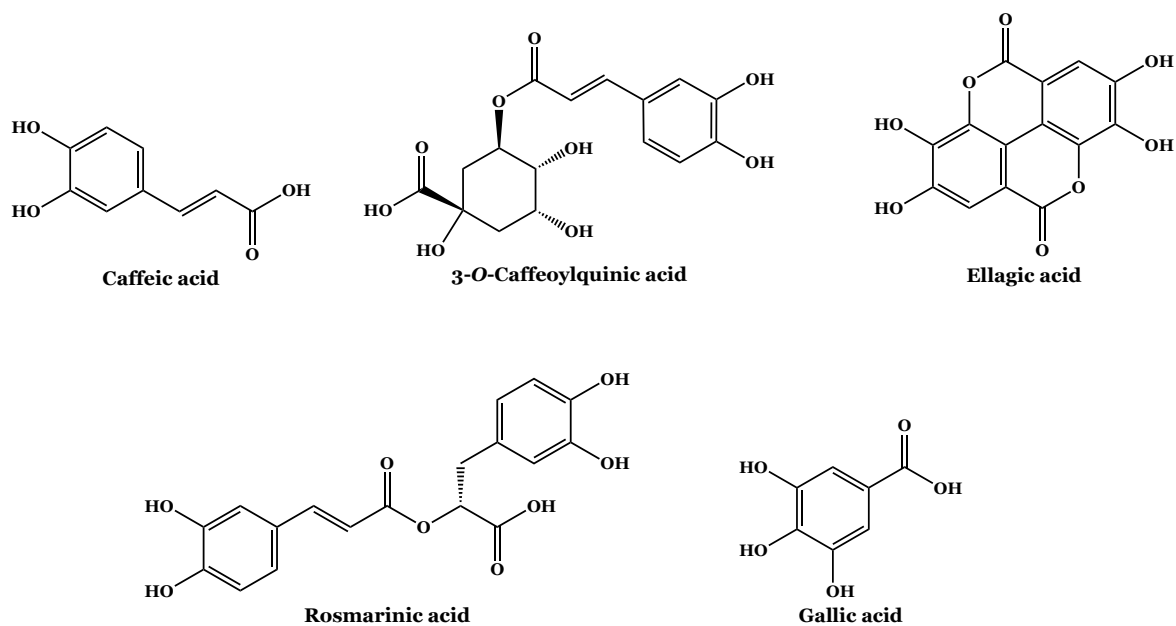


Figure 12. Structures of the phenolic acids identified in *T. catigua* stem bark extracts.

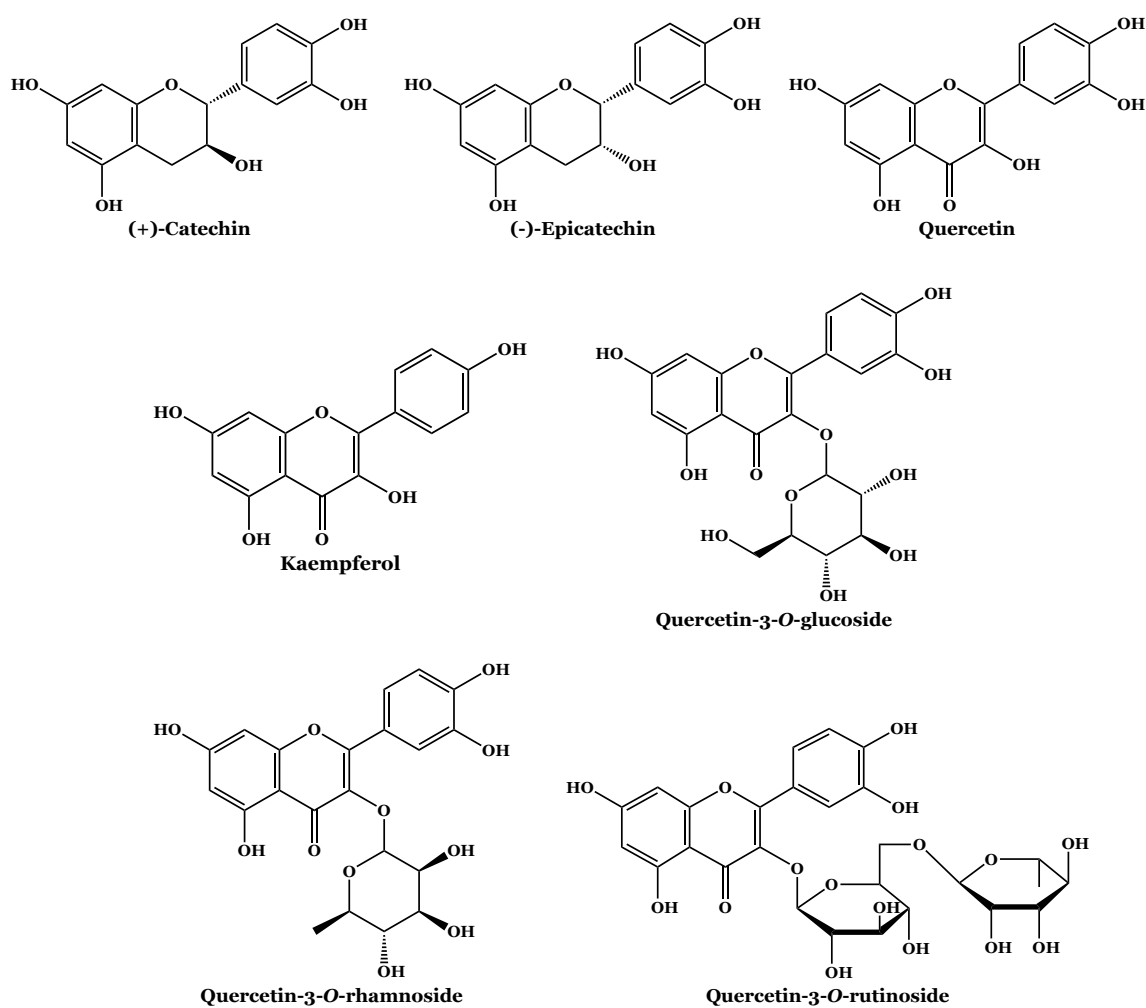


Figure 13. Structures of the flavonoids identified in *T. catigua* stem bark extracts.

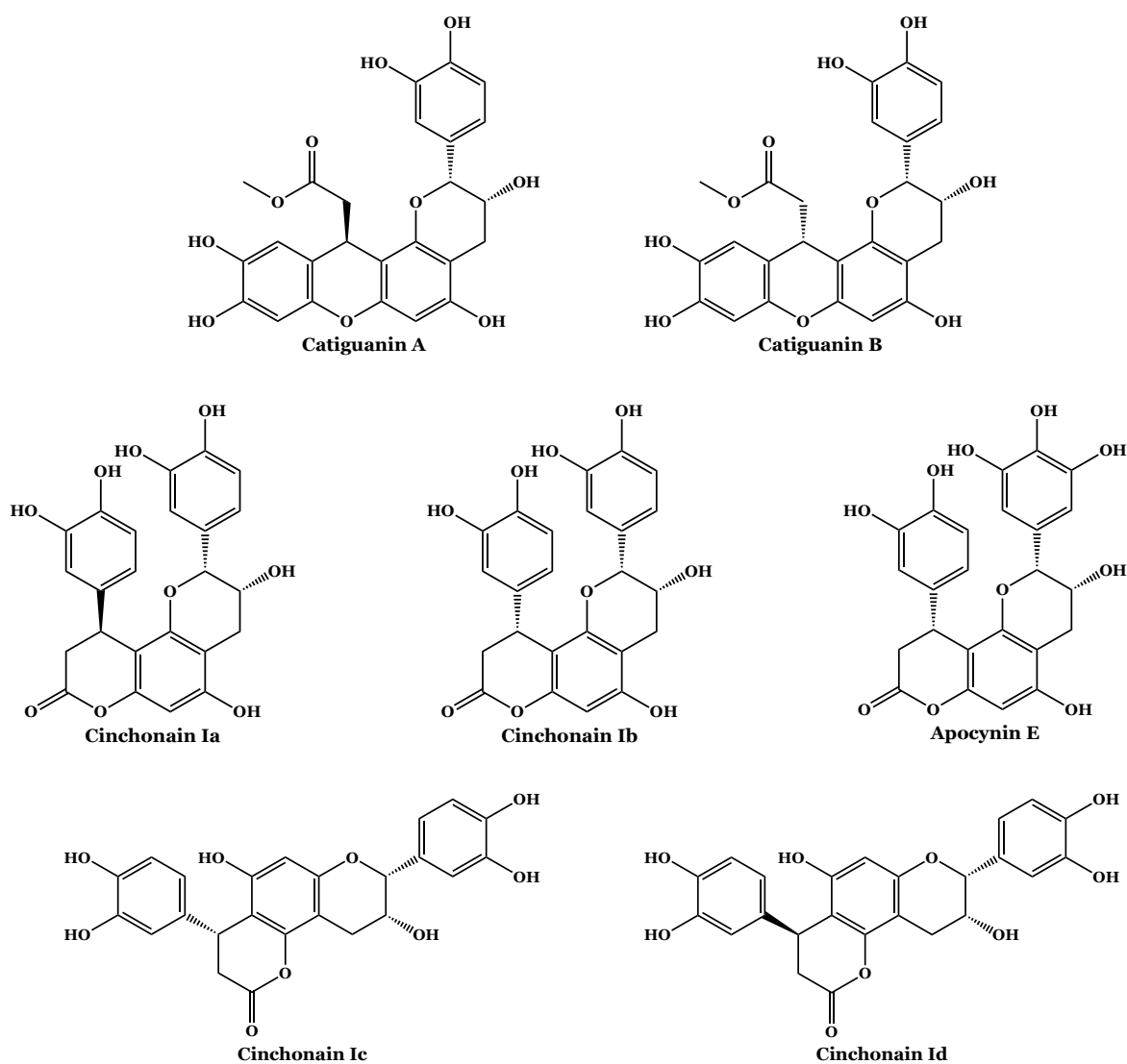


Figure 14. Structures of the phenylpropanoid-substituted flavan-3-ols identified in *T. catigua* stem bark extracts.

Cinchonain Ib (**Figure 14**) is used as chemotaxonomic marker to attest the botanical identity of *T. catigua* stem bark in TLC and HPLC quality control analysis of herbal products (342).

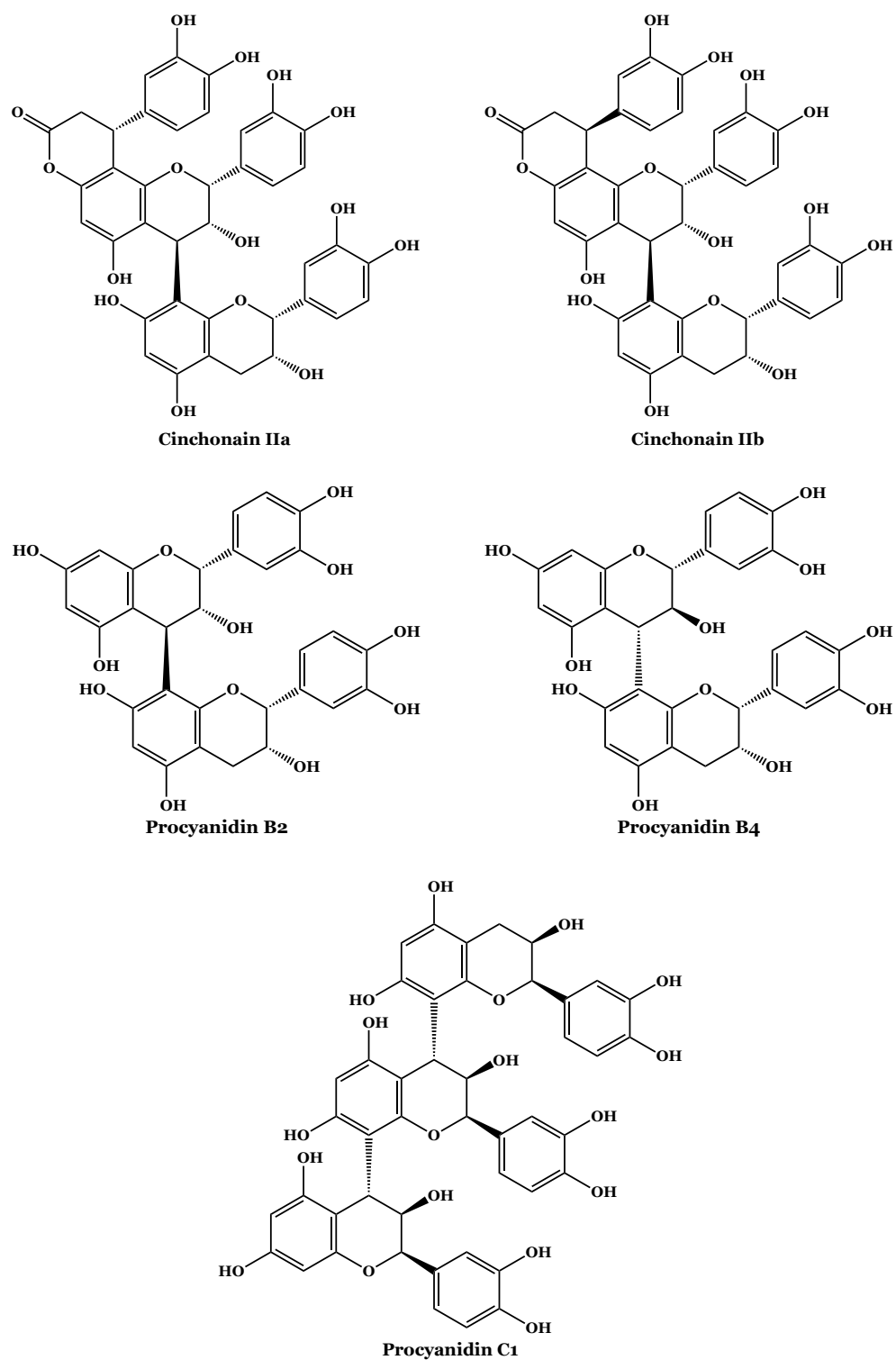


Figure 15. Structures of the procyanidins identified in *T. catigua* stem bark extracts.

1.3.1.2. Neuroprotective effects of *T. catigua* stem bark extracts

There is a growing body of scientific evidence to support the neuroprotective effects of *T. catigua* stem bark extracts.

These extracts exert *in vitro* anti-radical activity (335,336,338) and the ethyl acetate fraction displayed better antioxidant activity compared to the original acetone-water crude extract, which is supported by its also higher relative phenolic content (343). Therefore, with additional purification steps may be achieved a higher phenolic content and improved biological effects. From the six compounds isolated from a methanol extract (catiguanin A and B, and cinchonains Ia, Ib, Ic and Id) and tested for DPPH[•] radical scavenging activity, it was observed that cinchonains Ic and Id were the most effective (339). Furthermore, a hydro-ethanolic extract exerted neuroprotection against oxidative stress induced by different neurotoxic agents in rat hippocampal slices (337).

The acute and chronic oral treatments with an herbal product containing *T. catigua* (28.23%) resulted in a significant reduction of the immobility time in two models of depression in mice, comparable to that observed for classical antidepressant drugs and with possible modulation of the monoaminergic system (344). Ethyl acetate fractions of *T. catigua* extracts presented antidepressant-like effect, increased hippocampal cell proliferation and ameliorated memory in mice (345). In rodents was observed convincing evidence for a dopamine-mediated antidepressant-like effect of the bioactive compounds present in the hydroalcoholic extract of *T. catigua* stem bark (346).

The treatment of mice with an hydroalcoholic extract (cinchonain IIa, Ia and Ib as main constituents, and procyanidins) did not protect them from cold immobilization stress and scopolamine-induced amnesia. However, it partially reduced the fatigue induced by treadmill, since the highest dose increased the spontaneous locomotor activity and reduced the deficit on grip strength after forced exercise (347).

Herbal products containing *T. catigua*, as well as the hydroalcoholic extract alone, displayed antinociceptive effects in mouse nociception behavioural models, which were possibly associated with the activation of dopaminergic system and, to a lesser extent, to the interaction with the opioid pathway (348,349).

Another ethyl acetate fraction promoted functional recovery, decreased the delayed hippocampal cell loss, and mitigated the ongoing neurodegenerative processes in a mice model of cerebral ischemia (350). Furthermore, it was demonstrated that ethyl acetate fractions promoted functional recovery and restored hippocampal neurogenesis (351), and

they protected against oxidative stress and neuroinflammation after cerebral ischemia/reperfusion (352).

There is an ongoing phase II/III clinical trial to evaluate the therapeutic effects of escitalopram *versus* an association of escitalopram with *T. catigua* dry extract (LABCAT TCJUSS) with alteration on depression scores (353).

1.3.2. *Turnera diffusa* Willd. ex Schult.

Turnera diffusa Willd. ex Schult. (Turneraceae) is an endemic species of Mexico, Central and South America, and is popularly known as “damiana” (354). It is a small-branched shrub that grows from 0.3 up to 2 m height, with lance-shaped leaves that range between 10–25 mm in length, and has fragrant, small-rounded fruits and yellow conspicuous flowers that blossom in summer (355,356).

In traditional medicine, the stems, and leaves of *T. diffusa* (**Figure 16**) are used to prepare infusions, decoctions, and essential oils to stimulate the nervous system, as aphrodisiac, as diuretic, and for their hypoglycaemic effects and antimicrobial activity (355).



Figure 16. *T. diffusa* dried leaves (photography by João Bernardo).

1.3.2.1. Phenolic composition of *T. diffusa* leaves extracts

The total phenolic content of *T. diffusa* leaves is higher than that of stems (357). The main bioactive components identified in *T. diffusa* leaves extracts include simple phenolics, phenolic acids and flavonoids (**Figure 17**), mainly apigenin and its derivatives (**Figure 18**) and luteolin derivatives (**Figure 19**) (354).

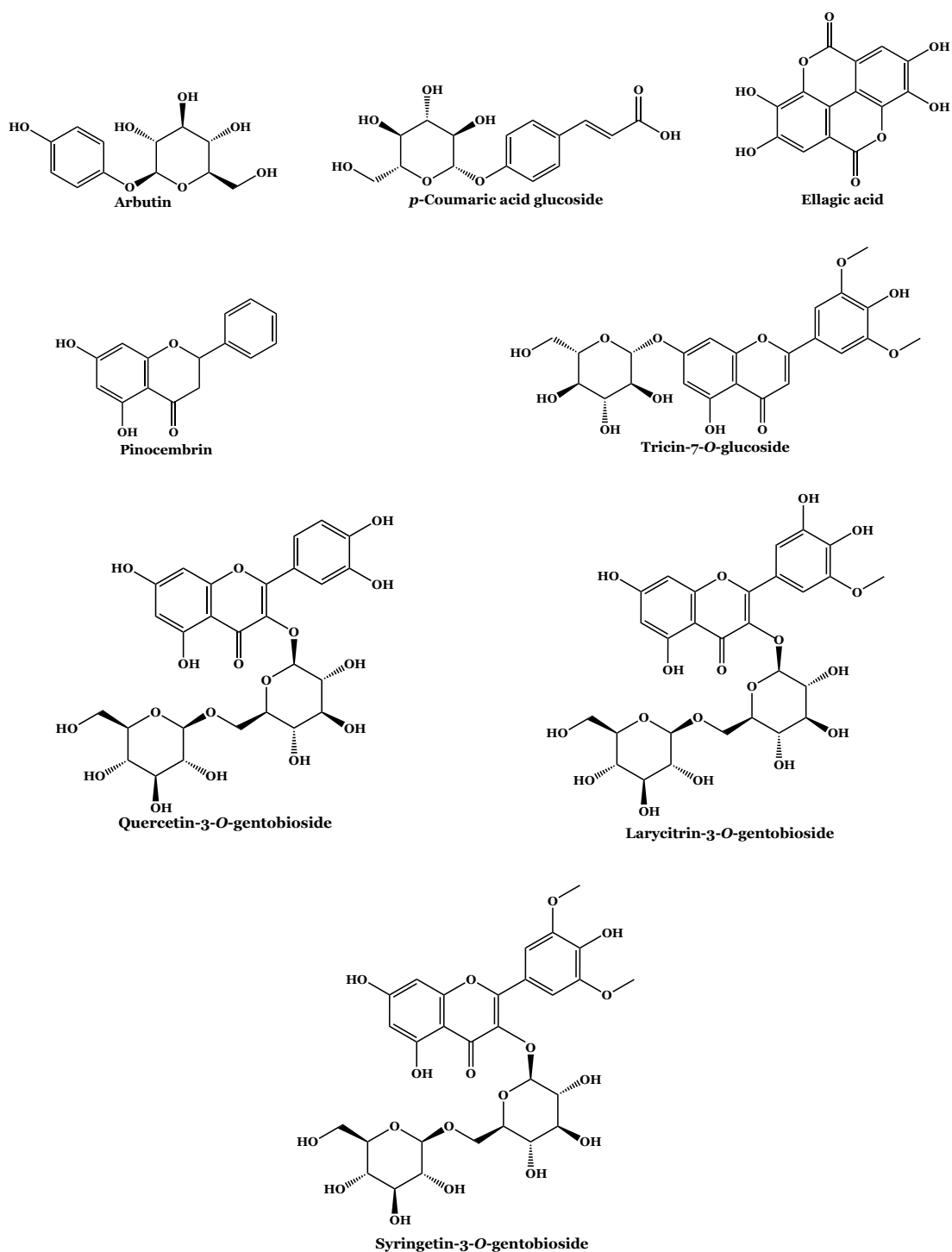
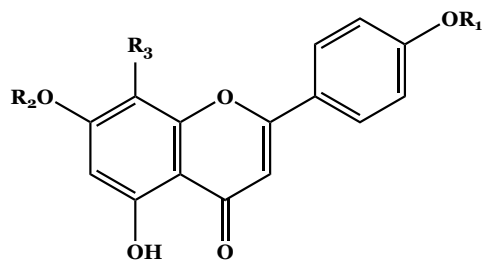
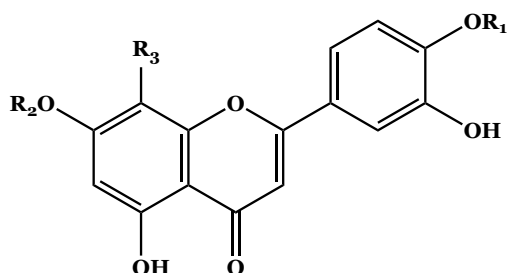


Figure 17. Structures of some phenolic compounds identified in *T. diffusa* leaves extracts.



	R₁	R₂	R₃
Apigenin	H	H	H
Apigenin-7-O-glucoside	H	Glucose	H
Apigenin-7-O-(4''-O-p-E-coumaroyl)-glucoside	H	<i>E</i> -coumaroyl-4-glucoside	H
Apigenin-7-O-(6''-O-p-E-coumaroyl)-glucoside	H	<i>E</i> -coumaroyl-6-glucoside	H
Apigenin-8-C-(2''-O-rhamnosyl)-glucoside	H	H	Rhamnosyl-glucoside
Acacetin	CH ₃	H	H
Acacetin-7-O-methyl ether	CH ₃	CH ₃	H

Figure 18. Structures of apigenin, its methoxy derivatives, and *O*- and *C*-glucosides identified in *T. diffusa* leaves extracts.



	R₁	R₂	R₃
Chrysoeriol-7-O-glucoside	CH ₃	Glucose	H
Velutin	CH ₃	CH ₃	H
Gonzalitosin I	CH ₃	CH ₃	OCH ₃
Luteolin-8-C-E-propenoic acid	H	H	<i>E</i> -propenoic acid
Luteolin-8-C-(2''-O-rhamnosyl)-glucoside	H	H	Rhamnosyl-glucoside
Luteolin-8-C-(2''-O-rhamnosyl)-quinovoside	H	H	Rhamnosyl-quinovoside
Luteolin-8-C-(6-deoxy-2''-O-rhamnopyranosyl)-xylo-hexopyranos-3-ulose	H	H	Deoxy-rhamnopyranosyl-xylo-hexopyranoside
Diosmetin-8-C-(2''-O-rhamnosyl)-glucoside	CH ₃	H	Rhamnosyl-glucoside

Figure 19. Structures of luteolin methoxy derivatives and *O*- and *C*-glucosides identified in *T. diffusa* leaves extracts.

The flavonoid luteolin-8-*C*-(6-deoxy-2"-*O*-rhamnopyranosyl)-*xyl*o-hexopyranos-3-uloside (**Figure 19**) is a chemotaxonomic marker used in quality control analysis of herbal products to attest the presence of this species (358,359).

1.3.2.2. Neuroprotective effects of *T. diffusa* leaves extracts

The supporting scientific evidence for the neuroprotective effects of *T. diffusa* leaves extracts is scarce. A phenolic-rich aqueous extract displayed *in vitro* anti-radical activity and prevented lipid peroxidation (357).

Acacetin-7-methyl ether (**Figure 18**) isolated from *T. diffusa* inhibited both MAO-A and MAO-B *in vitro*, but it was >500-fold selective against MAO-B over MAO-A and this inhibition was of the competitive type (360).

T. diffusa leaves extracts prepared with different extractants (petroleum ether, chloroform, methanol, and water) were tested for their anxiolytic-like effect in mice, using the elevated plus-maze apparatus. Only the methanol extract, (tested positive for alkaloids, cyanogenic glycosides, steroids, flavonoids, tannins, carbohydrates, and proteins) exhibited significant activity at a dose of 25 mg/kg with respect to control (361). This methanol extract was further fractionated with petroleum ether, chloroform, and butanol, and only the butanol fraction, which was composed by alkaloids and flavonoids, was found to exhibit the same effect (361).

T. diffusa hydroalcoholic solutions (85% ethanol) from different manufacturers displayed anxiolytic-like effect in mice (361). The aqueous extract of *T. diffusa* leaves induced an anxiolytic-like effect in rats (362), and anxiolytic-like and antidepressant effects in mice (363). In a study with bioactivity-guided fractions obtained from a *T. diffusa* methanol extract, apigenin was isolated and indicated as the compound responsible for the anxiolytic-like effect in mice using the elevated plus maze model of anxiety (364).

2. Objectives

The aim of this PhD thesis is to harness the neuroactive potential of medicinal plant extracts towards the production of new functional phyto-phospholipid nanoformulations.

The specific points are listed below:

- a) Characterization of the phenolic profile of *T. catigua* and *T. diffusa* aqueous extracts.
- b) *In vitro* study of the effects of *T. catigua* and *T. diffusa* extracts in selected targets and pathways engaged in aging-related chronic neurodegenerative disorders.
- c) To establish possible relationships between the bioactivities and phenolic composition of the respective extracts.
- d) Extraction of phospholipids for application as building-blocks in the development of phyto-phospholipid complexes.
- e) Production of phytosome nanoformulations with the flavonoid-rich extracts obtained from *T. catigua* and *T. diffusa*.
- f) Assessment of the impact of the flavonoid composition of the extracts in the physicochemical characteristics of the final nanoformulations.
- g) Evaluation of the performance of *T. catigua* and *T. diffusa* nanophytosomes in cell models to impair a neuroinflammatory stimulus and to protect neuronal cells from induced toxicity.

CHAPTER II

Experimental section

3. Experimental Section

3.1. Standards and reagents

The flavonoids acacetin, apigenin, apigenin-7-*O*-glucoside, apigenin-7-*O*-neohesperidoside, (+)-catechin, (-)-epicatechin, epigallocatechin, isorhamnetin 3-*O*-rutinoside, luteolin, luteolin-6-*C*-glucoside, luteolin-7-*O*-glucoside, luteolin-8-*C*-glucoside, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and vitexin-2-*O*-rhamnoside were from Extrasynthese (Genay, France). The reference standards ascorbic acid, 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, lutein and zeaxanthin were from Sigma-Aldrich (St. Louis, MO, USA). All the standards used in the identification and quantification of compounds were of HPLC grade ($\geq 95.0\%$).

All the following reagents used in the identification and quantification of compounds were of HPLC grade. Acetic anhydride and methanol were from Merck KGaA (Darmstadt, Germany), glacial acetic acid and ethanol were from Fisher Chemical (Pittsburgh, PA, USA), and acetone was from VWR (Lutterworth, UK).

The mixed standard solution of fatty acid methyl esters (FAME) (LCo8296V) composed of butanoic acid ($C_{4:0}$; CAS Number: 623-42-7), hexanoic acid ($C_{6:0}$; CAS Number: 106-70-7), octanoic acid ($C_{8:0}$; CAS Number: 111-11-5), decanoic acid ($C_{10:0}$; CAS Number: 110-42-9), undecanoic acid ($C_{11:0}$; CAS Number: 1731-86-8), dodecanoic acid ($C_{12:0}$; CAS Number: 111-82-0), tridecanoic acid ($C_{13:0}$; CAS Number: 1731-88-0), tetradecanoic acid ($C_{14:0}$; CAS Number: 124-10-7), *cis*-9-tetradecenoic acid ($C_{14:1n-5c}$; CAS Number: 56219-06-8), pentadecanoic acid ($C_{15:0}$; CAS Number: 7132-64-1), *cis*-10-pentadecenoic acid ($C_{15:1n-5c}$; CAS Number: 90176-52-6), hexadecanoic acid ($C_{16:0}$; CAS Number: 112-39-0), *cis*-9-hexadecenoic acid ($C_{16:1n-7c}$; CAS Number: 1120-25-8), heptadecanoic acid ($C_{17:0}$; CAS Number: 1131-92-6), *cis*-10-heptadecenoic acid ($C_{17:1n-7c}$; CAS Number: 75190-82-8), octadecanoic acid ($C_{18:0}$; CAS Number: 112-61-8), *cis*-9-octadecenoic acid ($C_{18:1n-9c}$; CAS Number: 112-62-9), *trans*-9-octadecenoic acid ($C_{18:1n-9t}$; CAS Number: 1937-69-8), *cis*-9,12-octadecadienoic acid ($C_{18:2n-6c}$; CAS Number: 112-63-0), *trans*-9,12-octadecadienoic acid ($C_{18:2n-6t}$; CAS Number: 2566-97-4), *cis*-6,9,12-octadecatrienoic acid ($C_{18:3n-6c}$; CAS Number: 16326-32-2), *cis*-9,12,15-octadecatrienoic acid ($C_{18:3n-3c}$; CAS Number: 301-00-8), eicosanoic acid ($C_{20:0}$; CAS Number: 1120-28-1), *cis*-11-eicosenoic acid ($C_{20:1n-11c}$; CAS Number: 2390-09-2), *cis*-11,14-eicosadienoic acid ($C_{20:2n-6c}$; CAS Number: 2463-02-7), *cis*-

8,11,14-eicosatrienoic acid ($C_{20:3n-6c}$; CAS Number: 21061-10-9), *cis*-11,14,17-eicosatrienoic acid ($C_{20:3n-3c}$; CAS Number: 55682-88-7), *cis*-5,8,11,14-eicosatetraenoic acid ($C_{20:4n-6c}$; CAS Number: 2566-89-4), *cis*-5,8,11,14,17-eicosapentaenoic acid ($C_{20:5n-3c}$; CAS Number: 2734-47-6), heneicosanoic acid ($C_{21:0}$; CAS Number: 6064-90-0), docosanoic acid ($C_{22:0}$; CAS Number: 929-77-1), *cis*-13-docosenoic acid ($C_{22:1n-9c}$; CAS Number: 1120-34-9), *cis*-13,16-docosadienoic acid ($C_{22:2n-6c}$; CAS Number: 61012-47-3), *cis*-4,7,10,13,16,19-docosahexaenoic acid ($C_{22:6n-3c}$; CAS Number: 2566-90-7), tricosanoic acid ($C_{23:0}$; CAS Number: 2433-97-8), tetracosanoic acid ($C_{24:0}$; CAS Number: 2442-49-1), and *cis*-15-tetracosenoic acid ($C_{24:1n-9c}$; CAS Number: 2733-88-2) was from Supelco (Bellefonte, PA, USA).

The enzymes AChE from *Electrophorus electricus* (EC 3.1.1.7), BuChE from equine serum (EC 3.1.1.8), human recombinant MAO-A (EC 1.4.3.4), 5-LOX from *Glycine max* L. (EC 1.13.11.12), tyrosinase from mushroom (EC 1.14.18.1) and XO from bovine milk (EC 1.1.3.22) were from Sigma-Aldrich.

Acetonitrile, acetylthiocholine iodide (ATCI), bovine serum albumin (BSA), Bradford reagent, aminoguanidine hydrochloride, boric acid, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH $\cdot$), methyl *t*-butyl ether (MTBE; HPLC grade), *S*-butyrylthiocholine iodide (BTCI), cholesterol ($\geq 99.0\%$), clorgyline ($\geq 97.0\%$), dimethyl sulfoxide (DMSO), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE; $\geq 97.0\%$), ether, ethylenedinitrilotetraacetic acid (EDTA), ethopromazine, formic acid, D-(-)-fructose ($\geq 99.0\%$), D-(+)-glucose ($\geq 99.5\%$), galantamine hydrobromide from *Lycoris* sp. ($\geq 94.0\%$), L-glutamic monosodium salt monohydrate ($\geq 98.0\%$), *n*-hexane, hydrochloric acid, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), interferon gamma (IFN- γ) from mouse, iron(II) sulphate heptahydrate, isopropanol, kojic acid ($\geq 98.5\%$), L-DOPA, D-mannitol, methanol, methylglyoxal solution, methylthiazolyldiphenyl-tetrazolium bromide (MTT), monobasic potassium phosphate, *N*-(1-naphthyl)ethylenediamine dihydrochloride, β -nicotinamide adenine dinucleotide reduced form (NADH), nitroblue tetrazolium chloride (NBT), perchloric acid, potassium dihydrogen phosphate, phenazine methosulfate (PMS), phosphoric acid, sodium azide, sodium chloride, sodium hydroxide, sodium nitroprusside dehydrate (SNP), sodium phosphate, sodium phosphate dibasic dihydrate, sodium sulphate anhydrous, sulphanilamide, sulphuric acid, sucrose, triethylamine, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), xanthine and kynuramine were also purchased from Sigma-Aldrich.

Ammonium molybdate was acquired to May & Baker Ltd. (London, UK). 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was from Alfa Aesar (Karlsruhe, Germany). The spectroscopic probe 16-(9-anthroyloxy)palmitic acid (16-AP) was from Molecular Probes, Inc (Plano, TX, USA). Linoleic acid was from VWR (Solon, OH, USA). Commercially available eggs (class L) from different batches of the same Portuguese supplier were used.

Dulbecco's Modified Eagle Medium (DMEM)+GlutaMAX, DMEM/Nutrient Mixture F-12 (DMEM/F12), Hank's Balanced Salt Solution (HBSS), heat inactivated fetal bovine serum (FBS), Pen-Strep solution (penicillin 5000 units/mL; streptomycin 5000 µg/mL) and trypsin-EDTA (at 0.25 and 0.05%) were purchased from Gibco (Invitrogen, Paisley, UK).

Human neuroblastoma SH-SY5Y cell line (CRL-2266TM) and mouse microglia BV-2 cell line (8EOC 2; ATCRL-2467) were acquired to American Type Culture Collection (ATCC; Manassas, Virginia, USA).

3.2. Plant material

T. catigua A.Juss. stem bark (lot 3553.204.13) and *T. diffusa* Willd. ex Schult. leaves (lot 3558.241.13) were acquired to Morais e Costa & CA. Lda (Porto, Portugal). The authenticity of the vegetal material was attested by the supplier's technical sheet and further ascertained by performing macroscopic and microscopic analyses following previous descriptions (333,355,356). Voucher specimens were deposited at herbarium of the Laboratory of Pharmacognosy of the Faculty of Pharmacy of University of Porto (FFUP), under the identification of TC-B-112016 and TD-L-092016, respectively, and then were stored in desiccators, in the dark and at room temperature, to avoid deterioration and physicochemical alterations.

3.3. *T. catigua* and *T. diffusa* crude aqueous extracts

3.3.1. Preparation of crude aqueous extracts

The plant material of each species was powdered and sieved ($\leq 910 \mu\text{m}$). The extracts were prepared by decoction of *ca.* 3 g of powdered material in 500 mL of water, for 20 min. The raw aqueous extracts were then filtered under vacuum, lyophilized (-45°C , 100 mT, for

5 days;) and finally stored in a dessicator protected from direct light exposure. The extraction yield was calculated as the amount of mass recovered following the equation: Extraction yield (%) = $100 \times (\text{mass obtained} / \text{mass of powdered material})$.

3.3.2. Phenolic profiling of crude aqueous extracts

3.3.2.1. *T. catigua*

3.3.2.1.1. Qualitative analysis by HPLC-DAD-ESI/MSⁿ

The qualitative chromatographic analysis by HPLC-DAD-ESI/MSⁿ was conducted in an Agilent HPLC 1100 series equipped with DAD and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an auto sampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, v. 08.03). The mass detector (model G2445A) was an ion trap spectrometer equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, v. .4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full scan mass covered the range from m/z 100 up to m/z 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionization mode. MSⁿ was carried out in the automatic mode on the more abundant fragment ion in MS⁽ⁿ⁻¹⁾.

The chromatography was developed on a Kinetex column (5 µm, C18, 100 Å, 150×4.6 mm; Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents, water-formic acid (1%) (A) and acetonitrile (B), in a gradient starting with 5% B to obtain 30% B at 30 min, and 50% B at 40 min. The flow rate was 1 mL/min and the injection volume of 20 µL. Data was recorded at 260, 280, 330 and 530 nm, and full scan mass covered the range from m/z 100 up to 1500.

3.3.2.1.2. Exact mass determination by UHPLC-ESI-QTOF-MS

UHPLC-ESI-QTOF/MS is a resourceful technique to achieve higher resolution and more accurate mass determinations (365). The determinations of exact mass were performed with an Agilent 1290 Infinity LC system coupled to the 6550 Accurate-Mass QTOF (Agilent Technologies, Waldbronn, Germany) with an electrospray interface (Jet Stream Technology). *T. catigua* crude extract was reconstituted (1 μ L) and injected in a reverse phase Luna Omega column (1.6 μ m, PS C18, 100 Å, 50 $\times$ 2.1 mm; Phenomex, Macclesfield, UK), protected with SecurityGuard ULTRA Cartridges of the same material (flow rate of 0.5 mL/min, 30 °C).

The mobile phase combined acidified water (0.1% formic acid; A) and acidified acetonitrile (0.1% formic acid; B). Compounds were separated with a gradient starting with 5% B to obtain 30% B at 12 min, and 50% B at 15 min. The optimal electrospray interface conditions were the following: gas temperature 280 °C; drying gas flow rate 11 L/min; nebulizer pressure 45 psi; sheath gas temperature 400 °C and sheath gas flow 12 L/min. The MS system was operated in negative ion mode with the mass ranging between m/z 50 and 1500, in full scan resolution mode.

3.3.2.1.3. Quantitative analysis by HPLC-DAD

The quantitative analysis was carried out with a HPLC-DAD unit (Gilson Medical Electronics, Villiers le Bel, France). The elution gradient and column were the same as the ones described above for the identification of compounds. *T. catigua* crude extract was reconstituted and filtered (0.45 μ m) before injection in the system (20 μ L). Detection was performed with an Agilent 1100 series DAD (Agilent Technologies, Waldbronn, Germany), and chromatograms were recorded at 280 and 320 nm (Clarity Software version 5.04.158, DataApex Ltd, Prague, Czech Republic). Reference compounds were analysed under the same conditions.

The compounds were quantified by interpolation of external standard curves (**Table 3**), as follows: 3- and 4-quinic acid esters (**1-3** and **6**) were quantified as 3-*O*-caffeoylquinic acid; 5-quinic acid esters (**5** and **7**) as 5-*O*-caffeyoilquinic acid; compound **25** was quantified as 3,5-*O*-dicaffeoylquinic acid; compounds **8** and **10** as epicatechin, and catechin (**4**) as itself; all the phenylpropanoid substituted flavan-3-ols (**9**, **10+11**, **12-24**, and **26**) were quantified as epigallocatechin.

Table 3. Linear regression equations, R^2 , limit of detection (LOD) and limit of quantification (LOQ) of the reference standards used in the identification and quantification of the phenolic compounds in *T. catigua* crude aqueous extract.

Reference standard	Linear regression	R^2	LOD ^a	LOQ ^a
3- <i>O</i> -Caffeoylquinic acid	$y = 30618.29x + 31.62$	0.999	7.55×10^{-3}	2.52×10^{-2}
5- <i>O</i> -Caffeoylquinic acid	$y = 37388.47x + 234.26$	0.998	1.11×10^{-2}	3.71×10^{-2}
3,5- <i>O</i> -Dicafeoylquinic acid	$y = 87086.53x + 652.08$	0.998	1.09×10^{-3}	3.63×10^{-3}
(+)-Catechin	$y = 24958.73x - 477.19$	0.997	2.00×10^{-4}	6.65×10^{-4}
(-)-Epicatechin	$y = 15904.36x + 27.83$	0.999	2.02×10^{-3}	6.73×10^{-3}
Epigallocatechin	$y = 3317.56x - 22.80$	0.998	1.99×10^{-2}	6.64×10^{-2}

^a mg/mL

3.3.2.2. *T. diffusa*

3.3.2.2.1. Extract hydrolysis

An additional step, consisting of acid hydrolysis followed by SPE clean-up, was applied to help in the identification of phenolic compounds present in the crude aqueous extract. Briefly, *T. diffusa* lyophilized crude extract was hydrolysed with HCl 2N (at 85 °C, 2 h) in a block heater apparatus (Stuart SBH130D, Staffordshire, UK). Then, the acidic solution was centrifuged (12000 rpm, 5 min) and cleaned-up with a C18 SEP-Pak cartridge (Waters, Milford, MA) that was previously activated with methanol and acid water (pH 2). The elution was performed with methanol, and the methanol solution was latter dried under vacuum (Savant SPD121P, Thermo Scientific). For analytical purposes, the residue was reconstituted in HPLC-grade water and filtered with a 0.22 µm size pore membrane.

3.3.2.2.2. Qualitative analysis by HPLC-DAD-ESI/MSⁿ

The chromatography was developed on a Kinetex column (5 µm, C18, 100 Å, 150×4.6 mm; Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents, water-formic acid (1%) (A) and acetonitrile-formic acid (1%) (B), in a gradient starting with 10% B to obtain 30% B at 20 min, and 60% B at 35 min. The flow rate was 1 mL/min and the injection volume of 20 µL. Spectral data was accumulated in the range of 240-400 nm and chromatograms were recorded at 330 nm.

3.3.2.2.3. Exact mass determination by UHPLC-ESI-QTOF/MS

The analysis was carried out with an Agilent 1290 Infinity LC system coupled to the 6550 Accurate-Mass QTOF (Agilent Technologies, Waldbronn, Germany) with an electrospray interface (Jet Stream Technology). *T. diffusa* hydrolysed extract (1 µL) was injected on a reverse phase Luna Omega column (1.6 µm, PS C18, 100 Å, 50×2.1 mm; Phenomenex, Macclesfield, UK) with SecurityGuard ULTRA Cartridges of the same material, operating at 30 °C and a flow rate of 0.5 mL/min.

The mobile phase consisted in acidified water (0.1% formic acid) (A) and acetonitrile (B). Compounds were separated starting with 10% B to obtain 40% B at 12 min, and 60% B at 13 min. The optimal conditions for the electrospray interface were 280 °C gas temperature, 11 L/min drying gas, 45 psi nebulizer pressure, 400 °C sheath gas temperature and 12 L/min sheath gas flow. The MS system operated in negative ion mode with the mass range set at m/z 50-1100 in full scan resolution mode.

3.3.2.2.4. Quantitative analysis by HPLC-DAD

The quantitative analysis was carried out with a HPLC-DAD unit (Gilson Medical Electronics, Villiers le Bel, France). The elution gradient and column were the same as the ones described above for the identification of compounds. *T. diffusa* crude extract was reconstituted and filtered (0.45 µm) before injection in the system (20 µL). Detection was performed with a Gilson DAD (Gilson Medical Electronics, Villiers le Bel, France), and data processed on Unipoint system software. Reference compounds were analysed under the same conditions and all chromatograms were recorded at 350 nm.

The compounds were quantified by interpolation of external standard curves (**Table 4**), as follows: apigenin-7-*O*-glucoside (**39**), quercetin-3-*O*-glucoside (**36**), luteolin (**49**), apigenin (**56**) and acacetin (**62**) were quantified as themselves; compound (**63**) was quantified as acacetin; the luteolin derivatives (**50–54**) as luteolin-7-*O*-glucoside, the 8-*C*-derivatives (**29**, **41** and **43–45**) as luteolin-8-*C*-glucoside, and 6-*C*-derivatives (**42** and **46**) as luteolin-6-*C*-glucoside; compounds **47+48** were quantified as apigenin-7-*O*-glucoside, while the other apigenin derivatives (**27**, **31**, **33**, **34**, **55** and **57–61**) as vitexin-2-*O*-rhamnoside; compound **37** was quantified as isorhamnetin-3-*O*-rutinoside; compounds **28**, **32** and **38** as myricetin-3-*O*-rhamnoside; and **30**, **35**, **39+40** as quercetin-3-*O*-rutinoside.

Table 4. Linear regression equations, R², limit of detection (LOD) and limit of quantification (LOQ) of the reference standards used in the identification and quantification of the phenolic compounds in *T. diffusa* crude aqueous extract.

Reference standard	Linear regression	R ²	LOD ^a	LOQ ^a
Acacetin	$y = 1.45 \times 10^9 x + 1.94 \times 10^5$	0.999	8.39×10^{-6}	2.80×10^{-5}
Apigenin	$y = 1.67 \times 10^9 x + 5.20 \times 10^5$	0.990	3.81×10^{-4}	1.27×10^{-3}
Apigenin-7- <i>O</i> -glucoside	$y = 8.19 \times 10^8 x + 3.03 \times 10^6$	0.992	1.57×10^{-4}	5.23×10^{-4}
Apigenin-7- <i>O</i> -neohesperidoside	$y = 1.01 \times 10^9 x + 5.24 \times 10^5$	0.999	3.30×10^{-5}	1.10×10^{-4}
Isorhamnetin-3- <i>O</i> -rutinoside	$y = 1.34 \times 10^9 x + 6.01 \times 10^6$	0.997	4.71×10^{-4}	1.57×10^{-3}
Luteolin	$y = 1.38 \times 10^9 x + 2.04 \times 10^6$	0.990	8.16×10^{-5}	2.72×10^{-4}
Luteolin-6- <i>C</i> -glucoside	$y = 7.15 \times 10^8 x + 2.84 \times 10^5$	0.998	5.41×10^{-4}	1.83×10^{-3}
Luteolin-7- <i>O</i> -glucoside	$y = 9.25 \times 10^8 x + 2.16 \times 10^5$	0.999	1.07×10^{-4}	3.56×10^{-4}
Luteolin-8- <i>C</i> -glucoside	$y = 6.07 \times 10^8 x + 2.15 \times 10^6$	0.998	5.41×10^{-4}	1.83×10^{-3}
Myricetin-3- <i>O</i> -rhamnoside	$y = 1.27 \times 10^9 x + 2.46 \times 10^6$	0.996	2.17×10^{-4}	7.22×10^{-4}
Quercetin-3- <i>O</i> -glucoside	$y = 8.47 \times 10^8 x + 1.44 \times 10^6$	0.995	1.57×10^{-4}	5.23×10^{-4}
Quercetin-3- <i>O</i> -rutinoside	$y = 2.31 \times 10^9 x + 1.91 \times 10^6$	0.999	9.61×10^{-5}	3.05×10^{-4}
Vitexin-2- <i>O</i> -rhamnoside	$y = 9.70 \times 10^8 x + 1.64 \times 10^6$	0.998	1.36×10^{-4}	4.55×10^{-4}

^a mg/mL

3.4. Lipid-based nanoformulations

3.4.1. Preparation and phenolic profiling of flavonoid-rich extracts

3.4.1.1. Clean-up by SPE

To produce nanophytosomes, the crude extracts of *T. catigua* and *T. diffusa* were subjected to an additional clean-up step by SPE with C18 (70 mL/ 10 000 mg) Chromabond® cartridges (Macherey-Nagel; Düren, Germany).

The lyophilized extracts (200 mg) were dissolved in 15 mL of water (pH 2.0). First, the column adsorbent was conditioned with methanol (1× column volume), followed by acidified water (2× column volume). The reconstituted extracts were then loaded, and washing was performed with acidified water (1× column volume). Methanol was the final elution solvent (4× column volume) that was latter evaporated under reduced pressure. The obtained phenolic-rich extracts were weighted. The extraction yield was calculated as the amount of mass recovered following the equation: Extraction yield (%) = 100× (mass obtained/mass of crude extract).

3.4.1.2. Phenolic profiling by HPLC-DAD

To validate the SPE procedure, and to ensure that the qualitative and relative quantitative phenolic composition of the flavonoid-rich extracts were the same as those of the initial crude extracts, the flavonoid-rich extracts were subsequently subjected to chromatographic analysis by HPLC-DAD. The conditions for each analysis were the same as described before (Section 3.3.2).

3.4.2. Phospholipid-rich fraction from egg yolk

3.4.2.1. Extraction procedure

The extraction of phospholipids from egg yolk was performed using ethanol and acetone as extraction solvents (**Figure 20**). Two egg yolks were used in each extraction process, and four independent extractions were performed. This procedure was rationalized considering the distinct solubility of neutral lipids and ionic phospholipids at room temperature, in organic solvents with different degrees of polarity, and taking advantage of the effect caused by the temperature drop to 4 °C on the solubility of lipids, in the solvents above mentioned (366,367). The final fraction, here designated as phospholipid-rich fraction, was stored at -20 °C, as dry extract or as EtOH/MeOH (2:1, v/v) solution, and protected from light, until further analysis.

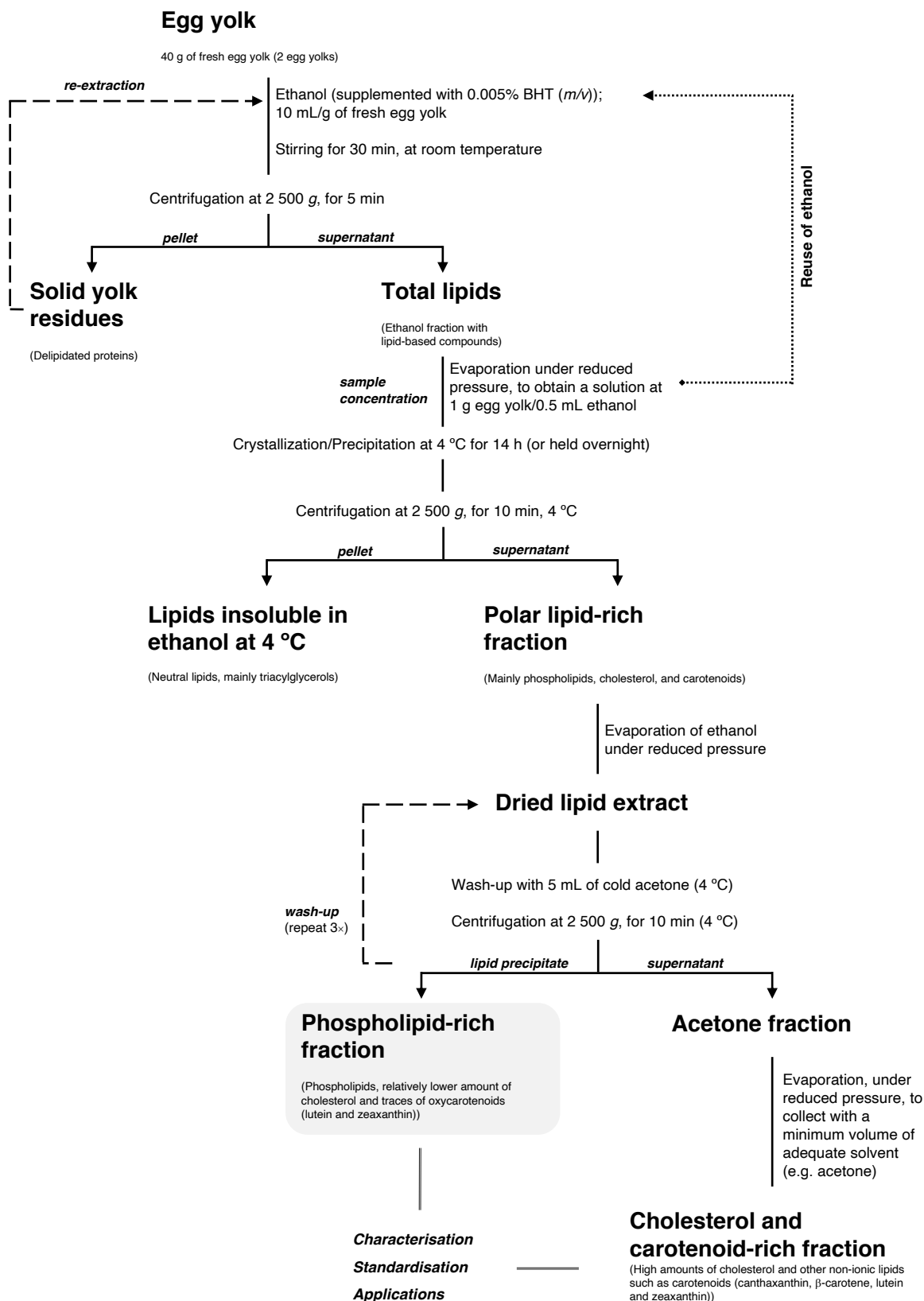


Figure 20. Flow diagram to obtain the phospholipid-rich fraction from egg yolk using ethanol and acetone as extractants.

3.4.2.2. Quantification of phospholipids

The quantification of was performed by first converting phospholipid phosphorus to inorganic phosphate, followed by the reaction with a molybdate salt to form a blue phosphomolybdic complex that can be spectrophotometrically quantified (368).

Aliquots of each phospholipid-rich fraction were collected and diluted (100×) with a solution of EtOH/MeOH (2:1; v/v). The diluted samples (25 µL) were dried under nitrogen stream, and then hydrolysed with 0.5 mL of perchloric acid 70%, at 180 °C, for 2 h, to release phosphates from phospholipids. Afterwards, 3.3 mL of H₂O, 0.5 mL of ammonium molybdate (0.25 g/10 mL) and 0.5 mL of ascorbic acid (1 g/10 mL) solutions were added to each glass tube, and the mixture was vortexed before incubation at 100 °C, for 10 min, in a water bath. The absorbance of the resulting phosphomolybdate complexes was determined at 800 nm. The phosphate concentration in each sample was calculated by interpolation of the external standard equation obtained with KH₂PO₄ (0–250 nmol) (**Table 5**), subjected to the same procedure as the samples.

Table 5. Linear regression equations, limit of detection (LOD) and of quantification (LOQ) of the external standards used for the chemical characterization of phospholipid-rich fractions.

	Linear regression	R ²	LOD	LOQ
Phosphates (nmol)	y= 0.0019x + 0.0569	0.999	22.579	68.421
Cholesterol (mg/mL)	y= 0.2682x + 0.0473	0.999	0.087	0.265
Lutein (mg/mL)	y= 327376x + 7.0302	0.999	7.13×10 ⁻⁵	2.16×10 ⁻⁴
Zeaxanthin (mg/mL)	y= 85718x + 14.1730	0.999	8.43×10 ⁻⁵	2.55×10 ⁻⁴

3.4.2.3. Quantification of cholesterol

The spectrophotometric quantification of cholesterol was achieved by the Liebermann-Burchard procedure (367), with minor alterations. Aliquots (25 µL) of the phospholipid-rich fraction in EtOH/MeOH (2:1; v/v) were collected to glass tubes, and solvents were evaporated under nitrogen stream. To the dried lipids were added 0.1 mL of acetic acid, and the reaction, at room temperature, was started by the addition of 5 mL of Liebermann-Burchard reagent (120 mL acetic anhydride + 60 mL acetic acid + 20 mL sulphuric acid + 4 g Na₂SO₄). After 30 min, the absorbance of the resulting blue-green complex was determined at 630 nm. The analytical concentrations were calculated by interpolation of the external standard equation of cholesterol (0.31–2.50 mg/mL) (**Table 5**), subjected to the same procedure as the samples.

3.4.2.4. Identification and quantification of carotenoids by HPCL-DAD

Aliquots (250 μ L) of phospholipid-rich fraction in EtOH/MeOH (2:1; V/V) were collected, and solvents were evaporated under nitrogen stream. Carotenoids were extracted from the dried lipid extracts by addition of 10 mL of acetone. The mixture was sonicated (15 min) in a water ultrasonic bath (88/320 W, 35 kHz; Bandelin Sonorex Digitec), followed by stirred maceration (45 min, at 600 rpm). The acetone extracts were collected and filtered through nylon membranes (0.22 μ m pore size), and then the solvent was evaporated under reduced pressure. The dried extract was stored at -20 °C until further analysis.

To proceed with the identification and quantification of carotenoids, the dried extracts were solubilized in 200 μ L of acetone, from which 20 μ L were injected in the HPLC-DAD unit (Gilson Medical Electronics, Villiers le Bel, France). Analytical samples were eluted using a C30 YMC column (5 μ m, 250 $\times$ 4.6 mm; YMC, Kyoto, Japan), with temperature stabilization at 25 °C. The mobile phase consisted of methanol (A) and MTBE (B), and the elution gradient started with 95% A to obtain 70% A at 30 min, finishing with 50% A at 50 min. The flow rate was set at 0.9 mL/min. Detection was performed with an Agilent 1100 series DAD (Agilent Technologies, Waldbronn, Germany) and data analysed with Clarity Software (version 5.04.158, DataApex Ltd, Prague, Czech Republic). Chromatograms were recorded at 450 nm and compared with those of the external standards lutein and zeaxanthin. Their content was calculated by interpolation of the respective external standard equations (**Table 5**).

3.4.2.5. Characterization of phospholipid classes by TLC

Phospholipid classes from the obtained fractions were separated by TLC using silica gel plates (TLC Silicagel 60 F₂₅₄; Merck KGaA, Germany). Prior to separation, the plates were activated with chloroform/methanol (1:1, v/v) and treated with boric acid (2.3% in ethanol). The plates containing independent spots of samples (20–30 μ g) or standards (5 μ g) were developed in the solvent mixture of chloroform:ethanol:water:triethylamine (35:30:7:35, v/v/v/v). Lipids spots on TLC were detected at 365 nm after revelation with primuline (50 μ g/100 mL, in acetone:water (80:20, v/v)). Pure Egg PC and DPPE were used as reference standards since lecithin and phosphatidylethanolamines are the major classes in egg yolks.

3.4.2.6. Preparation of methyl esters of fatty acyl chains and profiling by GC-FID

Fatty acid methyl esters from the dried lipids extracts were obtained by acid catalysed transmethylation, as described elsewhere (368). Aliquots (900 nmol phospholipids) from each PL-rich fraction were placed in glass tubes with 30 μ L of the internal standard (IS; fatty acid C₁₇ 1 mM, dissolved in chloroform), and the mixture dried under a nitric stream. The dried extract was dissolved in 5 mL of freshly prepared HCl/MeOH solution (5%), and then incubated in a thermostatic water bath at 70 °C for 2 h. After cooling to room temperature, 5 mL of *n*-hexane were added to each test tube and the mixture was vortexed and centrifuged (3 min, 2000 rpm.). The upper phase, the hexane fraction containing fatty acid methyl esters, was collected and the lower phase was re-extracted with an equal volume of *n*-hexane. The combined fractions were mixed with 6 mL of H₂O and subjected to a new centrifugation step (3 min, 2000 rpm). Next, the aqueous phase was discharged and the water remaining in the hexane phase was removed by the addition of sodium sulphate anhydrous (*ca.* 1 g). Finally, the solution was filtered, and the solvent evaporated under reduced pressure. The extracted methyl esters were solubilized with 200 μ L of *n*-hexane and stored at -20 °C, until further analysis.

The fatty acid profiling was carried out in a Finnigan Focus gas chromatography (GC) apparatus (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a flame ionization detector (FID) and a VF-5 MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) (Varian BV, Middelburg, Netherlands). The injection volume was of 1 μ L (derivatized lipid extracts containing the IS, and commercial FAME mixture). The carrier was high purity helium C-60 (Gasin, Portugal) at constant 1 mL/min flow rate. Temperatures of the injector and detector were maintained at 250 °C. The oven temperature was set at 40 °C for 1 min, then increased 5 °C/min to 250 °C, 3 °C/min to reach 300 °C and held for 15 min. The compounds were identified by comparing their retention times with the ones of FAME mixture, and their content calculated as relative % of the IS.

3.4.3. Preparation and characterization of nanoformulations

All lipid-based formulations, nanoliposomes and nanophytosomes, were prepared following the method of thin-film hydration, followed by extrusion (**Figure 21**). In general, aliquots of phospholipid-rich fractions were collected and dissolved in 5 mL of ethanol/methanol (2:1, v/v) in round-bottom flasks. Then solvents were evaporated to dryness under reduced pressure in a rotary evaporator with controlled temperature (140

rpm, at 37°C). The resulting dried lipid film was hydrated with 10 mL of suspension buffer (NaCl 50 mM + HEPES 10 mM, pH 7). In the next step, the mixture was subjected to sonication cycles (2 min) in alternation with vortex shaking (2 min), in a total of 20 min, at controlled temperature (37 °C). Finally, the suspensions of multilaminar small vesicles were manually extruded through polycarbonate membranes (200 nm pore size). Non-entrapped compounds were removed through dialysis using a cellulose membrane (cut-off 14 kDa; Sigma-Aldrich), for 18 h with suspension buffer, and then properly stored.

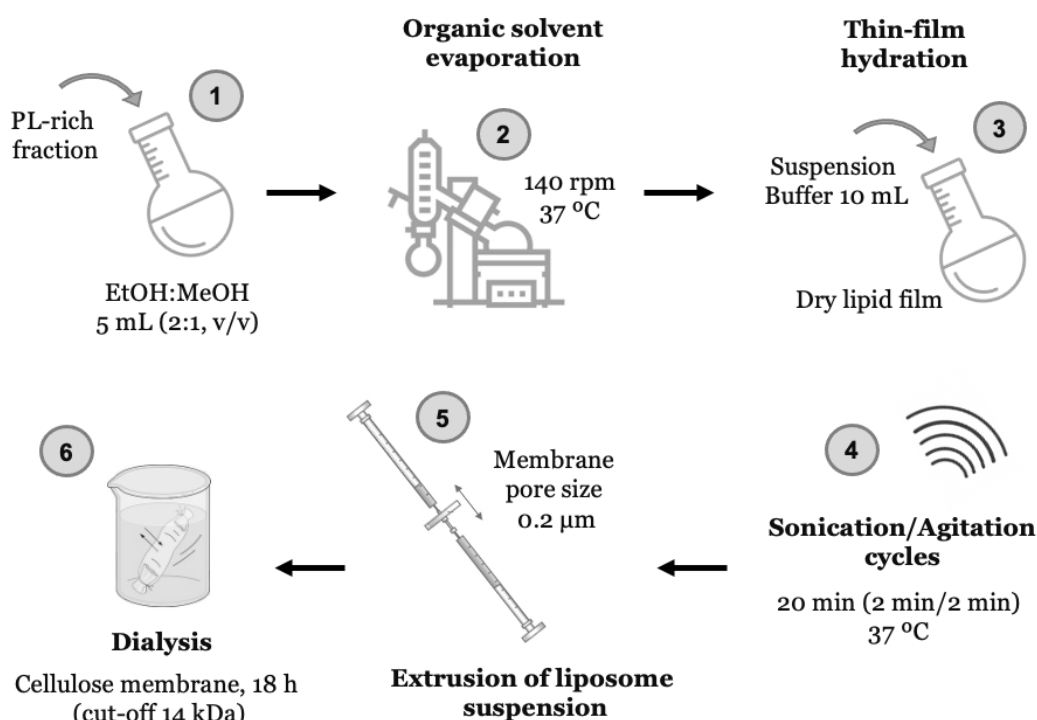


Figure 21. Outline of the protocol for preparation of nanoliposomes and nanophytosomes with the phospholipid (PL)-rich fraction.

Rutin nanoliposomes, and respective blank controls, were prepared by following the procedure described above. The lipid content was set at 2.5 mM phospholipid, and the suspension buffer (10 mL) was supplemented with rutin to obtain the concentrations of 250, 125, 63 and 31 μM at the final formulations.

T. catigua and *T. diffusa* nanophytosomes, and blank nanoliposomes, were prepared at 5 mM and 900 μM phospholipids. The suspension buffer (10 mL) was supplemented with the flavonoid-rich extracts of each species, and the phospholipids:extract molar *ratio* was set to 97:3 in *T. catigua* phytosomes (900 μM phospholipids + 6 μg/mL of extract; 5 mM

phospholipids + 33 µg/mL of extract), and to 70:30 in *T. diffusa* phytosomes (900 µM phospholipids + 85 µg/mL of extract; 5 mM phospholipids + 472 µg/mL of extract).

Simultaneously, for the study of the stability profiles, blank nanoliposomes, and *T. catigua* and *T. diffusa* nanophytosomes were prepared at 5 mM phospholipid, while keeping the same phospholipids:phenolics molar *ratios*. In addition, to produce lyophilized formulations, part of the original corresponding ones was collected and subjected to dialysis with suspension buffer supplemented with mannitol (20%), and then lyophilized.

3.4.3.1. Entrapment efficiency

UV–Visible spectra of non-dialyzed and dialyzed nanoformulations were recorded in the range of 250–650 nm with 1 nm of resolution (Thermo Spectronic Unicam Helios Alpha UV–Visible spectrophotometer).

The absorption band with maximum at 350 nm was used to determine the entrapment efficiency (EE) of rutin, and at 280 nm for *T. catigua* and *T. diffusa* flavonoid-rich extracts. EE represents the fraction of rutin, or flavonoid-rich extract, incorporated into liposomes when compared with the total amounts initially added to the suspension buffers. It was calculated following the equation: $EE (\%) = \frac{Abs \text{ post dialysis}}{Abs \text{ before dialysis}} \times 100$.

3.4.3.2. Particle size and Zeta potential

All samples were diluted in suspension buffer (1:4) to yield a suitable scattering intensity (count rate between 300 and 500 kcps). Particle size, size distribution and polydispersity index (PDI) were assessed by dynamic light scattering in a particle size analyser (Brookhaven Instruments, Holtsville, NY, USA). Individual running time was of 120 s, and 6 runs *per* measurement were performed.

Zeta potential was determined by electrophoretic light scattering (ELS), using a ZetaPALS instrument (Brookhaven Instruments, Holtsville, NY, USA). Data was recorded from six runs (10 cycles per run) with a relative residual value (measure of data fit quality) of 0.03.

3.4.3.3. Incorporation of 16-AP probe in liposomes and determination of the partition coefficient of rutin

16-AP probe was added to previously prepared liposome suspensions (100, 300, 400 and 600 μM PL) to obtain the final concentration of 0.65 μM . To promote the incorporation of 16-AP into the lipid bilayers, the mixtures were placed under magnetic stirring for 4 h at 37 °C and protected from light exposure. Then, suspension buffer supplemented with rutin was added to aliquots of each liposome suspension (rutin at final molarities of 0, 1, 3, 8, 21, 35 and 50 μM), while keeping the lipid molarity constant. Afterwards, the mixtures were again subjected to magnetic stirring for 4 h at 37 °C.

The location of rutin within the liposome lipid bilayer and its lipid-water partition were determined by its quenching effect on the fluorescence of 16-AP. The fluorescence emission spectra of 16-AP ($\lambda_{\text{exc}} = 360$ nm), in the absence and presence of rutin, was acquired between 390 and 650 nm. The lipid-water partition coefficient was determined from the Stern-Volmer modified equation as described by Fato and collaborators (369), and following the equation: $\frac{1}{K_{app}} = \left(\frac{1}{K_q} - \frac{1}{K_q K_p} \right) m + \frac{1}{K_q K_p}$, where K_{app} is the apparent quenching constant determined experimentally by Stern-Volmer plots, K_q is the biomolecular quenching constant for rutin in the membrane, K_p is the partition coefficient, and α_m is the volume fraction of the membrane phase assuming a lipid-specific volume of 0.984 $\mu\text{L}/\text{mg}$ of lipid (370).

3.4.3.4. Transmission electron microscopy analysis

For transmission electron microscopy (TEM) *T. catigua* and *T. diffusa* nanophytosomes, as well as blank nanoliposomes, were deposited on Formvar-carbon coated grids (TAAB Laboratories, Aldermaston, England) for 5 min, and then contrasted with uranyl-acetate 2% for 1 min. Observations were carried out at 100 kV, using a Tecnai G2 Spirit BioTwin transmission electron microscope (FEI Company, Hillsboro, NE, USA).

3.4.3.5. Accelerated stability and demixing behaviour

The study of accelerated stability and demixing behaviour of lipid nanoformulations was conducted with a multisampling analytical photocentrifuge LUMiSizer® (L.U.M GmbH, Berlin, Germany). Blank liposomes and *T. catigua* and *T. diffusa* nanophytosomes,

as well as the corresponding lyophilized versions, were prepared at 5 mM phospholipid. Briefly, 600 μ L of each sample were centrifuged at 25 °C, for 29 min at 4000 rpm, by using capped disposable rectangular polycarbonate cells (path length of 2 mm) (L.U.M. GmbH PC 110-131XX), which were placed horizontally into the photocentrifuge. During centrifugation the emitted near-infrared light (NIR-light) passed through the cell and a charge-coupled device line detector (CCD-line detector), and was recorded at 5 s intervals, in a total of 350 transmission profiles at a specific wavelength (λ = 865 nm). Data was presented as function of the radial position. Data visualization and the calculation of the average migration velocity and instability index were assessed using the SEPView® 6.3 software (L.U.M GmbH, Berlin, Germany).

3.5. *In vitro* evaluation of biological activities

3.5.1. Antioxidant activity

3.5.1.1. Total antioxidant capacity assay

The total antioxidant capacity (TAC) was evaluated by reduction of molybdenum (VI) to molybdenum (V) by the sample analyte and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH (**Figure 22**) (371). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in deionized water (final concentrations [15–600] μ g/mL). In test tubes, 100 μ L sample solutions were mixed with 900 μ L of reagent solution (H_2SO_4 0.6 M + Na_3PO_4 28 mM + $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 4 mM). The reaction was developed in a water bath at 95 °C, for 90 min. After cooling to room temperature, aliquots of 200 μ L were collected and placed in 96-wells plate and the absorbance was measured at 695 nm. Ascorbic acid was assayed under the same conditions. TAC results were expressed as μ g/mL of ascorbic acid equivalents (AAE) by interpolation of the linear regression equation: $y = 0.02646x + 0.08047$.

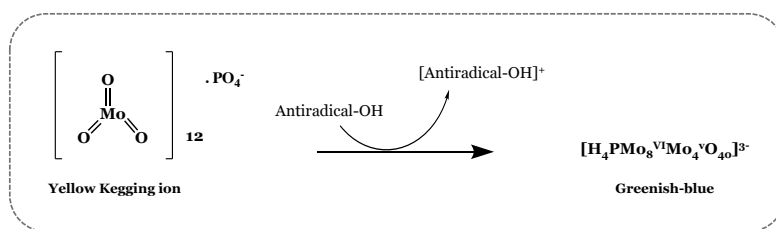
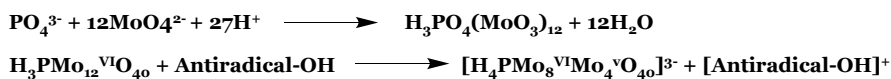


Figure 22. Chemical reactions in total antioxidant capacity assay.

3.5.1.2. Scavenging of DPPH• assay

The scavenging of DPPH• was determined as function of its reduction to formazan crystals (**Figure 23**) (372). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in water (final concentrations [3–250] µg/mL), and 25 µL were added to each well. The reaction started with the addition of 200 µL of DPPH• methanol solution (150 µM). The 96-wells plate was incubated at room temperature for 30 min, protected from light. After this period, the absorbance was recorded at 515 nm in a Multiskan GO plate reader (Thermo Fisher Scientific; Waltham, MA, USA) and compared with that obtained for controls (without crude extracts) and respective blanks (without DPPH• methanol solution). Results were expressed following the equation:

$$\text{DPPH radical scavenging (\%)} = 100 - \left(\frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control} - \text{Abs blank}} \times 100 \right).$$
 Ascorbic acid was the positive control ($\text{IC}_{50} = 6.77 \pm 0.10 \text{ µg/mL}$).

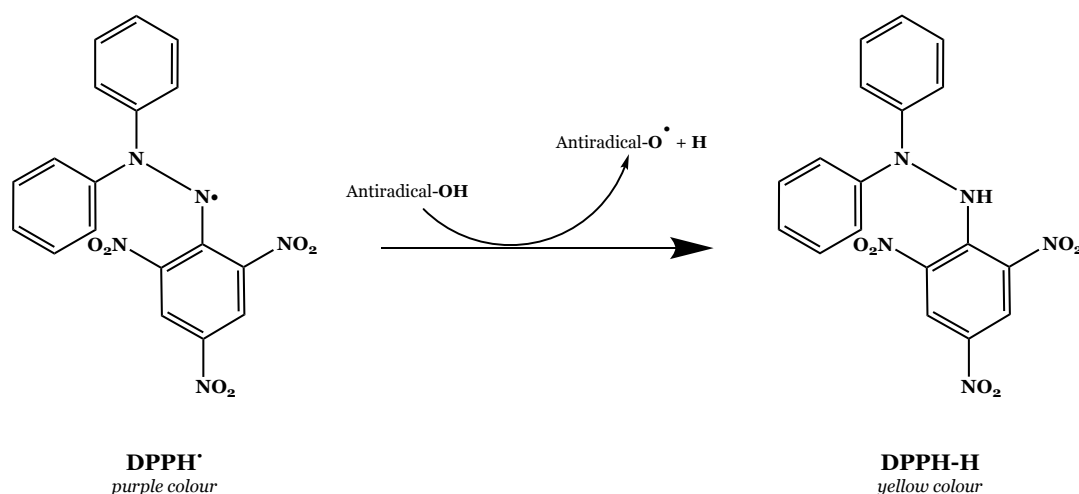


Figure 23. Reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to 2,2-diphenyl-1-picrylhydrazine (DPPH-H) by antiradical hydrogen transfer.

3.5.1.3. Scavenging of $\cdot\text{NO}$ assay

SNP was used to spontaneously generate $\cdot\text{NO}$, and NO_2^- was spectrophotometrically determined by the Griess reaction (**Figure 24**) (373). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in KH_2PO_4 buffer (100 mM, pH 7.4; final concentrations [0.01–2.5] mg/mL), and 100 μL were added to each well. Afterwards, to each well were added 100 μL of SNP 20 mM and the 96-wells plate were incubated for 60 min, at room temperature, under light exposure. Finally, 100 μL of Griess reagent (1% sulphanilamide + 0.1% N-(1-naphthyl) ethylenediamine, in 2% phosphoric acid) were added and the mixture was incubated at room temperature, for 10 min. The absorbance was determined at 562 nm, in a plate reader, and compared with that obtained for controls (without crude extracts) and respective blanks (2% phosphoric acid). Results were expressed following the equation:

$\text{NO radical scavenging (\%)} = 100 - \left(\frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control} - \text{Abs blank}} \times 100 \right)$. Quercetin was the positive control ($\text{IC}_{50} = 40.03 \pm 12.90 \mu\text{g/mL}$).

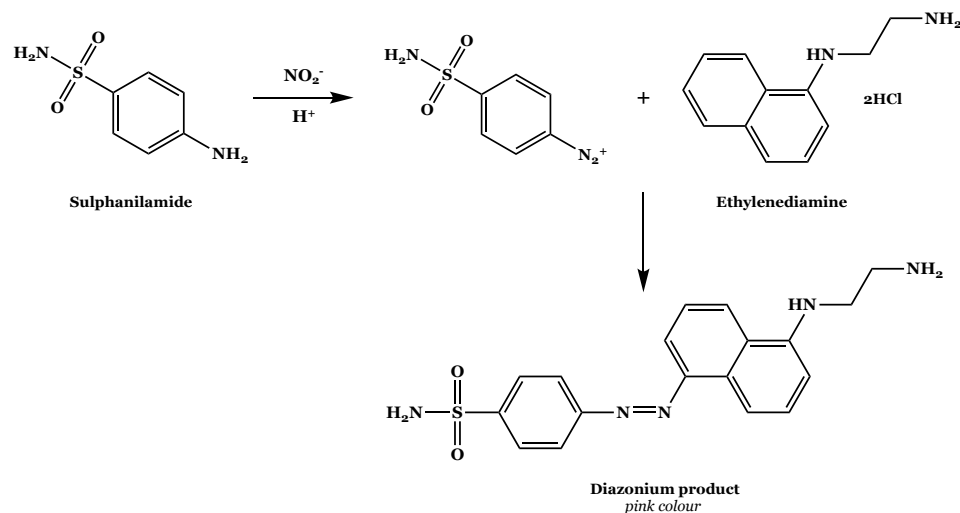


Figure 24. Determination of nitrite by Griess reaction.

3.5.1.4. Scavenging of $\text{O}_2^{\cdot-}$ assay

The scavenging of $\text{O}_2^{\cdot-}$ was assessed based on the generation of superoxide radicals by the non-enzymatic PMS/NADH system, and subsequent reduction of NBT by the radical into blue-coloured diformazan (**Figure 25**) (374). Serial dilutions of *T. catigua* and *T.*

diffusa crude extracts were prepared in KH_2PO_4 buffer (19 mM, pH 7.4; final concentrations [3–850] $\mu\text{g/mL}$), and 50 μL were added to each well. Afterwards, to each well were added 50 μL of NADH solution (166 μM), 150 μL of NBT solution (43 μM) and finally 50 μL of PMS solution (2.7 μM). The reaction was started with the addition of PMS. The rate was determined in kinetic mode in a 96-wells plate reader, for 2 min at 560 nm, and compared to that of controls (without crude extracts). Results were expressed following the equation:

$$\text{Superoxide anion radical scavenging (\%)} = 100 - \left(\frac{\text{Abs sample}}{\text{Abs control}} \times 100 \right).$$

Quercetin was the positive control ($\text{IC}_{50} = 24.24 \pm 0.85 \mu\text{g/mL}$).

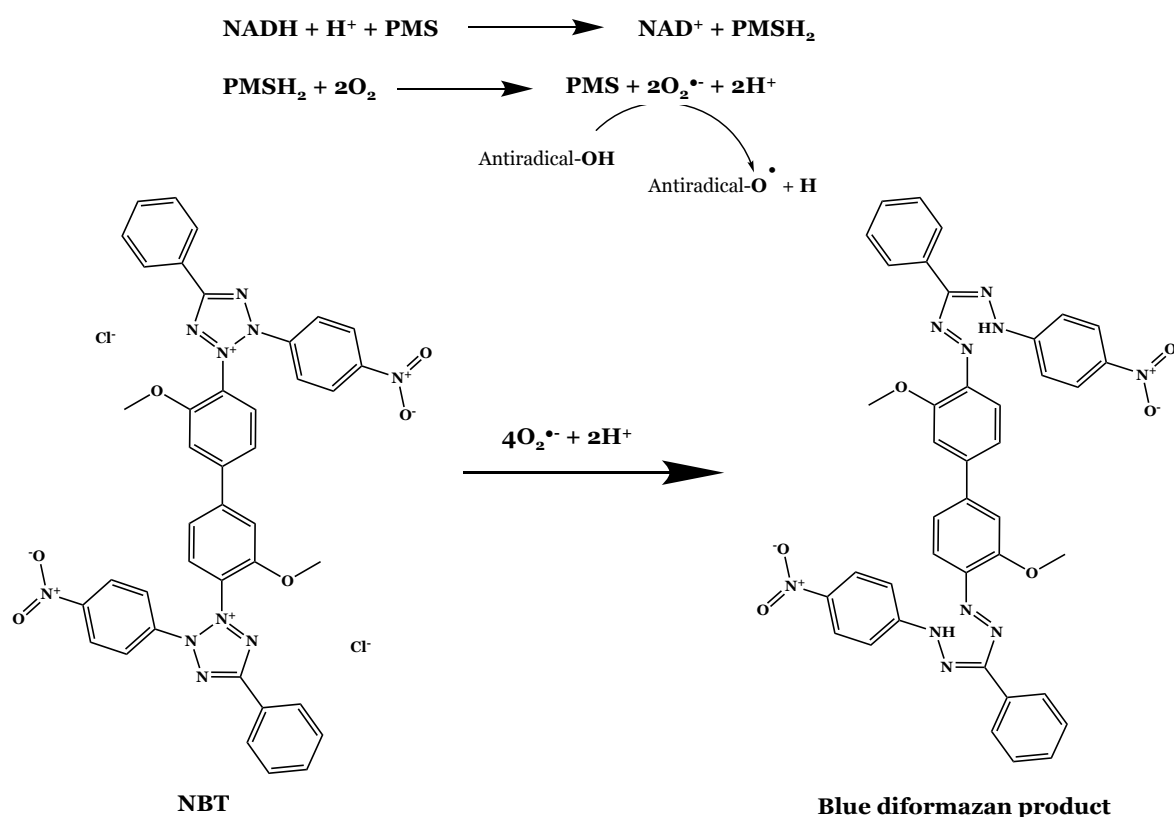


Figure 25. Superoxide anion radical scavenging assay. Superoxide anion radical is formed by the non-enzymatic NADH/Phenazine methosulfate (NADH/PMS) system. The free radical reacts with nitroblue tetrazolium chloride (NBT) to form a diformazan product.

3.5.1.5. Linoleic acid peroxidation assay

The peroxidation of linoleic acid was determined following the formation of conjugated dienes (**Figure 26**) triggered by Fe^{2+} /ascorbic acid (375). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in Tris-HCl buffer (0.1 M, pH 7.5; final concentrations [9–600] $\mu\text{g/mL}$). In test tubes, the reaction mixture contained 25 μL of test

samples, 75 μL of reaction buffer, 125 μL of linoleic acid ethanol solution (20 mM) and 25 μL of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (4 mM). The reaction started with the addition of 25 μL of ascorbic acid (5 mM), and tubes were incubated for 60 min, at 37 $^\circ\text{C}$. Ended this period, 725 μL of ethanol:ether (3:1, v/v) were added to each test tube. The absorbance was measured at 233 nm in a spectrophotometer and compared with that obtained for controls (without crude extracts) and blanks (ethanol). Results were expressed following the equation: $\text{Linoleic acid peroxidation (\%)} = \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control}} \times 100$. BHT was the positive control ($\text{IC}_{50} = 404.19 \pm 24.18 \mu\text{g/mL}$).

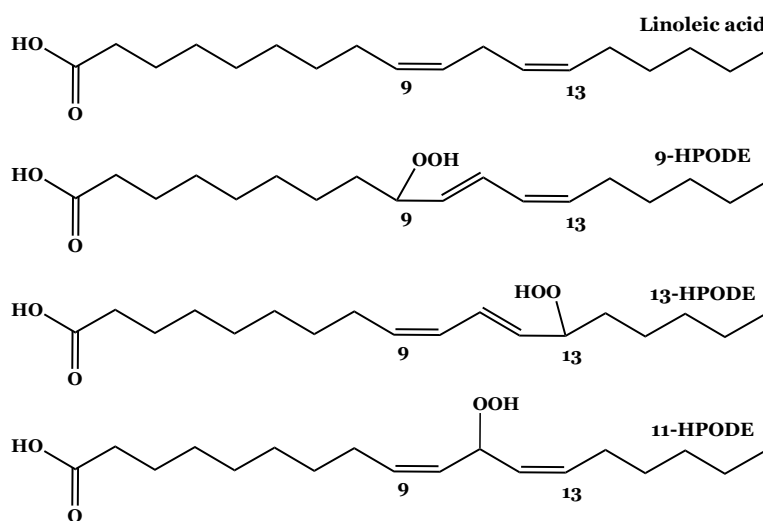


Figure 26. Structures of linoleic acid and of its major conjugated hydroperoxides, 9-hydroperoxyoctadecadienoic acid (9-HPODE), 13-hydroperoxyoctadecadienoic acid (13-HPODE) and 11-hydroperoxyoctadecadienoic acid (11-HPODE).

3.5.2. BSA-glycation assays

The extent of protein glycation was assessed by non-enzymatic reactions between reducing sugars and BSA to form fluorescent cross-linking AGEs (376).

Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ buffer (0.2 M, pH 7.4, containing NaN_3 0.2 mg/mL; final concentrations [5–150] $\mu\text{g/mL}$). Test tubes contained 25 μL of sample solutions, 150 μL of reaction buffer and 75 μL of BSA (50 mg/mL). The reaction started after addition of 125 μL of one glycation inducer (glucose at 800 mM, fructose at 300 mM, or methylglyoxal at 15 mM), and tubes were maintained at constant temperature (37 $^\circ\text{C}$). Aliquots of 200 μL were collected at four time-points (0, 3, 7 and 14 days). The fluorescence ($\lambda_{\text{exc}} = 360 \text{ nm}$ / $\lambda_{\text{em}} = 435 \text{ nm}$) was determined in a spectrofluorimeter (SynergyTM HT, Biotek Instruments Winooski, USA).

For each glycation inducer the results were expressed following the equation:

$$AGEs \text{ (fold increase)} = \frac{\text{Fluorescence (t0,3,7 or 14)}}{\text{Fluorescence (t0)}}. \text{ Aminoguanidine was the positive control.}$$

3.5.3. Enzymatic assays

3.5.3.1. AChE and BuChE assays

The activities of cholinesterase enzymes were assessed based on Ellman's method (**Figure 27**) (377). The following buffers were prepared: buffer A composed by 50 mM Tris-HCl, pH 8; buffer B composed by 50 mM Tris-HCl + 0.1% BSA (w/v), pH 8; buffer C composed by 50 mM Tris-HCl + 0.1 M NaCl and 0.02 M MgCl₆H₂O, pH 8.

Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in buffer A (final concentrations [150–3000] µg/mL). Lyophilised AChE was dissolved in buffer A (1000 U/mL, stock solution), and further diluted with buffer B (0.44 U/mL). In each well were mixed 25 µL of test samples, 50 µL of buffer B, 25 µL of ATCI (15 mM, in water) and 125 µL of DTNB (3 mM, in buffer C). The reaction started with the addition of 25 µL of AChE (final concentration of 0.44 U/mL). The rate was determined in kinetic mode in a 96-wells plate reader, for 2 min at 405 nm, and compared to that of controls (without crude extracts).

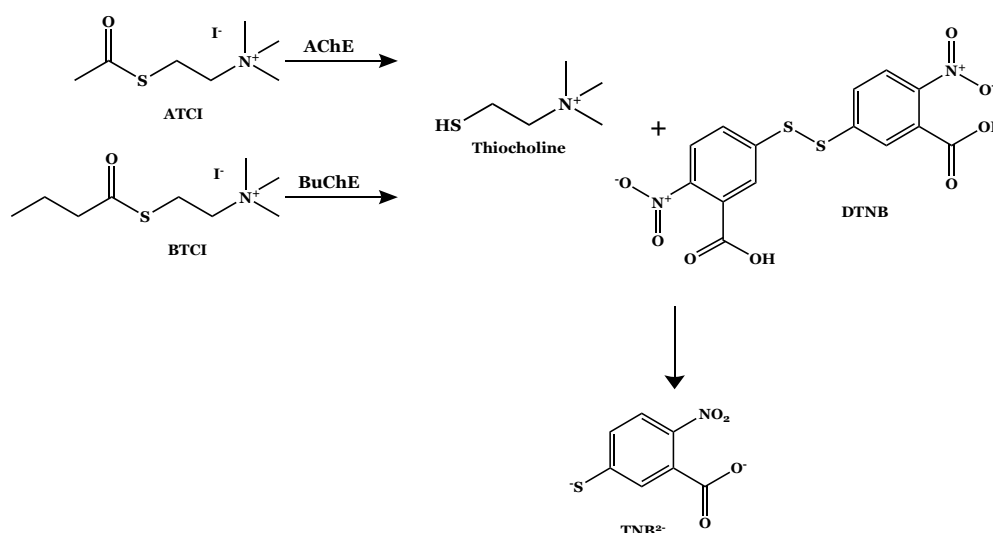


Figure 27. Reactions underlying Ellman's method. Thiocholine results from the hydrolysis of acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCl) by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), respectively, and then it reacts with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to generate the anion TNB with yellow colour.

The activity of BuChE enzyme was determined as described before, with minor alterations. The enzyme was BuChE (in buffer A, final concentration of 0.1 U/mL) and the substrate was BTCI (15 mM, in water). Results were expressed following the equation: $\text{Cholinesterase inhibition (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$. Galantamine was the positive control in AChE ($\text{IC}_{50} = 2.11 \pm 0.15 \mu\text{g/mL}$) and BuChE ($6.16 \pm 0.29 \mu\text{g/mL}$) assays.

3.5.3.1.1. AChE inhibition in rat brain homogenates

The biological material, brains of male Wistar rats, was kindly provided by the Laboratory of Toxicology of FFUP. The animals were used in accordance with the internationally accepted principles of laboratory animal use and care, as found in the European Community guidelines, and approved by the FFUP-ORBEA Ethical Committee.

AChE activity in rat brain tissue homogenates was also determined based on Ellman's method. Serial dilutions of *T. catigua* lyophilized crude extracts (final concentrations of 200, 100 and 50 $\mu\text{g/mL}$) were prepared in buffer A. Briefly, brain homogenates (1:10, m/v) were prepared with a cold working solution (250 mM sucrose + 0.5 mM EDTA + 10 mM HEPES, pH 7.4). After centrifugation (14 000 *g*, 10 min at 4° C) the enzymatic activity was determined in the supernatant by the catalytic over ATCI (0.15, 0.30, 1.5, 2.0, 3.0, 10.0, 15.0 and 20.0 mM), monitored in kinetic mode (increasing absorbance at 450 nm, 2 min at 37° C) in a microplate reader. To avoid cross reactions, the experiments were conducted in the presence of the BuChE selective inhibitor ethopromazine (0.1 mM). The protein content was determined with the Bradford reagent at 595 nm in a microplate reader, with BSA as reference.

3.5.3.2. MAO-A assay

MAO-A activity was assessed in terms of catalytic deamination of kynuramine to 4-hydroxyquinoline (**Figure 28**) (378). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts were prepared in potassium phosphate buffer (0.1 M, pH 7.4; final concentrations [10–650] $\mu\text{g/mL}$). In test tubes, the reaction mixture contained 5 μL of test samples, 350 μL of reaction buffer and 125 μL of MAO-A (17 U/mL). The reaction started with the addition of 20 μL of kynuramine (3.75 mM) and was developed for 70 min, at 37 °C. Finally, the reaction was stopped 75 μL NaOH (2N). The absorbance was determined at 314 nm in

a spectrophotometer and compared to that obtained for controls (without crude extracts) and blanks. Results were expressed following the equation:

$MAO - A \text{ inhibition (\%)} = \frac{Abs \text{ control} - Abs \text{ sample}}{Abs \text{ control}} \times 100$. Clorgyline was the positive control ($IC_{50} = 0.035 \pm 0.005 \mu\text{g/mL}$).

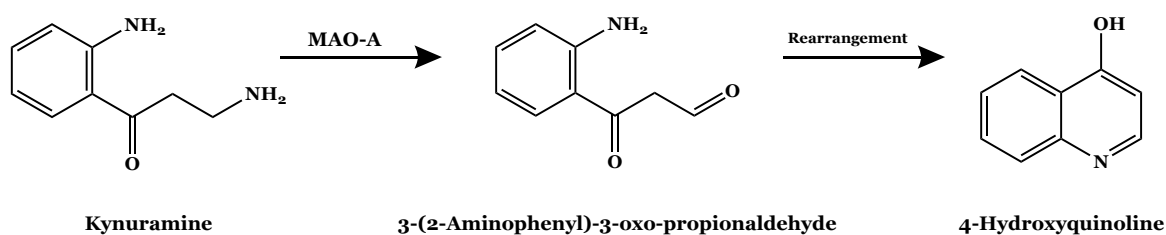


Figure 28. Metabolism of kynuramine to 4-hydroxyquinoline by monoaminooxidase-A (MAO-A).

3.5.3.3. XO assay

The activity of XO was assessed in terms of formation of uric acid from the metabolism of xanthine (**Figure 29**) (379). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts (final concentrations [1-250] $\mu\text{g/mL}$) were prepared in reaction buffer (50 mM KH_2PO_4 + 5 mM EDTA, pH 7.4). In each well, the reaction mixture was composed by 20 μL of test sample, 200 μL of reaction buffer and 20 μL of XO (0.5 U/mL). The reaction was started with the addition of 20 μL of xanthine (2.5 mM). The reaction rate was determined in kinetic mode in a 96-wells plate reader, for 2 min at 295 nm, and compared to that of controls (without crude extracts). Results were expressed following the equation: $XO \text{ inhibition (\%)} = \frac{Abs \text{ sample}}{Abs \text{ control}} \times 100$. Allopurinol was the positive control ($IC_{50} = 18.88 \pm 0.94 \mu\text{g/mL}$).

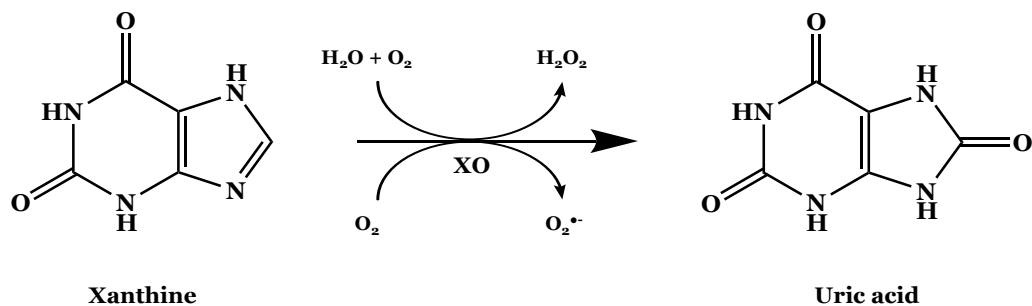


Figure 29. Metabolism of xanthine to uric acid by xanthine oxidase (XO).

3.5.3.4. Tyrosinase assay

The diphenolase activity of tyrosinase was assessed by using L-DOPA as substrate, with resulting formation of dopachrome (**Figure 30**) (380). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts (final concentrations [25–1600] µg/mL) were prepared in reaction buffer (0.05 M KH₂PO₄, pH 6.8). In each well, the reaction mixture consisted of 40 µL of test solutions, 80 µL of reaction buffer and 40 µL of tyrosinase (84 U/mL). The reaction started with the addition of 40 µL of L-DOPA (4.5 mM). The rate was determined in kinetic mode in a 96-wells plate reader (37 °C), for 3 min at 475 nm, and compared to that of controls (without crude extracts). Results were expressed as inhibition of tyrosinase activity following the equation: $Tyrosinase\ inhibition\ (\%) = 100 - \left(\frac{Abs\ sample}{Abs\ control} \times 100 \right)$. Kojic acid was the positive control (IC₅₀ = 1.93 ± 0.16 µg/mL).

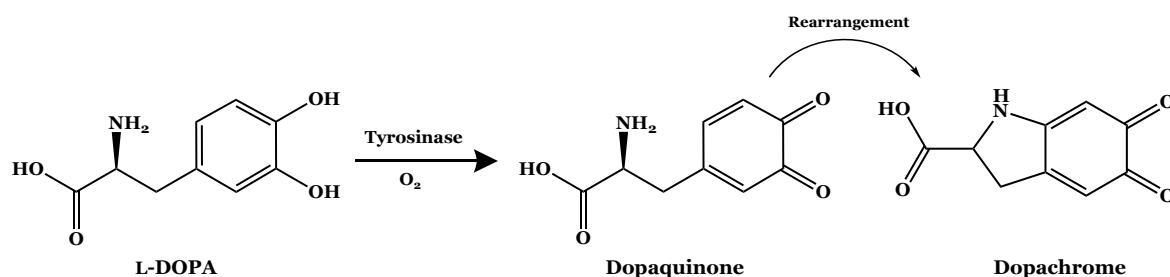


Figure 30. Metabolism of L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase and formation of dopachrome.

3.5.3.5. 5-LOX assay

The activity of 5-LOX was studied following the oxidation of linoleic acid (**Figure 31**) (381). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts (final concentrations [3–400] µg/mL) were prepared in reaction buffer (0.1 M Na₂HPO₄·2H₂O, pH 9.0). In each well, the reaction mixture consisted of 20 µL of sample solutions, 200 µL of reaction buffer and 20 µL of enzyme (100 U). The reaction started with the addition of 20 µL of linoleic acid (4.18 mM in ethanol). The rate was determined in kinetic mode in a 96-wells plate reader, for 3 min at 234 nm, and compared to that of controls (without crude extracts). Results were expressed as inhibition of enzyme activity (%) following the equation: $5LOX\ inhibition\ (\%) = 100 - \left(\frac{Abs\ sample}{Abs\ control} \times 100 \right)$. Quercetin was the positive control (IC₅₀ = 1.77 ± 0.24 µg/mL).

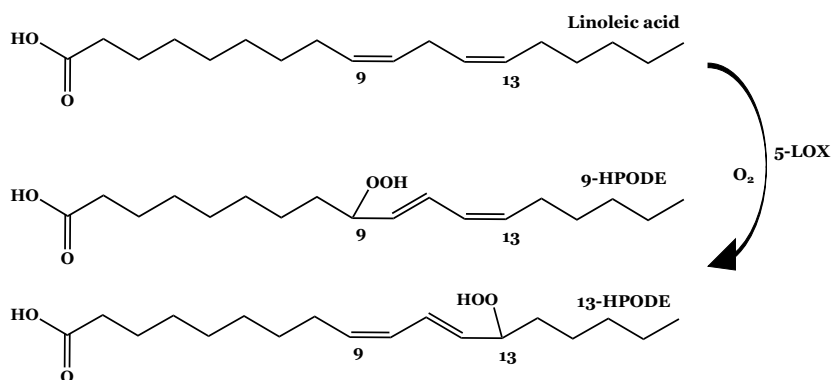


Figure 31. Oxidation of linoleic acid by 5-lipoxygenase (5-LOX).

3.5.4. Cell assays

3.5.4.1. Assays with SH-SY5Y cell line

3.5.4.1.1. Cell culture conditions

The human neuroblastoma SH-SY5Y cell line was routinely cultured, and maintained, with the culture medium DMEM/F12 (supplemented with 10% FBS and 1% Pen Strep). Cells were kept in a controlled humidified atmosphere of 5% CO_2 , at 37 °C, in 75 cm³ culture flasks. When cells reached a near-confluent stage, the monolayer was detached with a solution of 0.05% Trypsin-EDTA (3 mL) and collected to a sterile conical centrifuge tube. Culture medium (10 mL) was also added, and the cell suspension was centrifuged (1100 rpm, 3 min). Then, the culture medium was discarded, and the cell pellet suspended in culture medium (6 mL). To determine cell density, cells were manually quantified in a Neubauer chamber with trypan-blue exclusion test.

3.5.4.1.2. Cell viability

Cell viability was assessed in terms of MTT reduction by mitochondrial dehydrogenases of metabolically active cells to form an insoluble purple formazan product (**Figure 32**) (382). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts, blank nanoliposomes, rutin-nanoliposomes and *T. catigua* and *T. diffusa* nanophytosomes were all prepared with

culture medium and sterilized by passing through a 0.22 μm pore size membrane. SHSY-5Y cells were seeded at 40000 cells/well in 96-wells plates and treated for 24 h. After this incubation period, the culture medium was discarded and replaced by 0.5 mg/mL MTT solution (prepared in culture medium) and cells were incubated for another 2 h. Finally, the culture medium was removed from each well and formazan crystals solubilized in DMSO:isopropanol (3:1, v/v). The absorbance was determined at 510 nm in a plate reader and compared to that from respective controls. Results were expressed following the equation: $\text{Cell viability (\%)} = \frac{\text{Abs sample}}{\text{Abs control}} \times 100$.

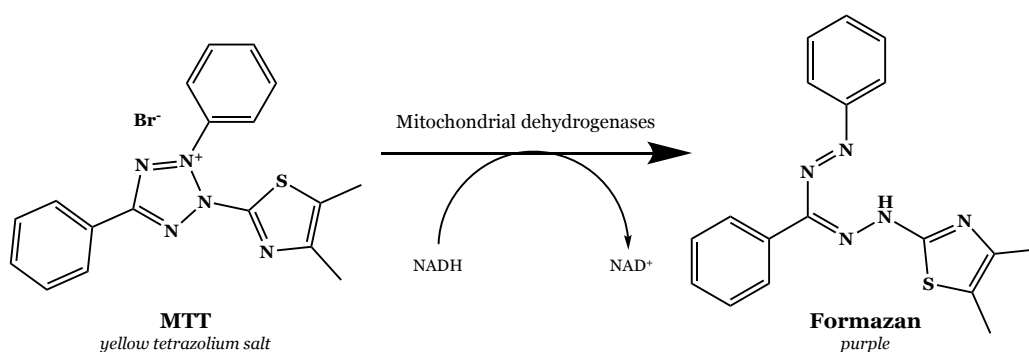


Figure 32. Reduction of methylthiazolyldiphenyl-tetrazolium bromide (MTT) to formazan crystals by mitochondrial dehydrogenases of metabolically active cells.

3.5.4.1.3. Toxic stimuli

SH-SY5Y cells were treated with two toxicants, 6-OHDA/AA (100 mM + 0.02% AA) or glutamate (50 mM), in separate assays. SH-SY5Y cells were seeded in DMEM/F12 at 40000 cells/well in 96-wells plates. Some cells were left without treatment (controls), while others were incubated for 22 h with a toxicant.

In addition, to evaluate the protective effects of the test samples (*T. catigua* and *T. diffusa* crude extracts, blank nanoliposomes, rutin-nanoliposomes, and *T. catigua* and *T. diffusa* nanophytosomes) against the cytotoxic effects, SH-SY5Y cells were also pre-treated with the test samples for 3 h, followed by a 22 h period of co-incubation with either 6-OHDA/AA or glutamate. After 24 h the cell viability was assessed in terms of MTT reduction, as detailed above.

3.5.4.1.4. Determination of intracellular reactive species

SH-SY5Y cells were seeded at 40 000 cells/well in dark 96-wells plates. First, cells were incubated for 1 h with DCFH-DA (25 mM, prepared in culture medium). After this period,

the over plus of fluorescent probe not incorporated was washed out with HBSS. Then, some cells were left without treatment (controls), while the toxic stimulus was induced by glutamate (50 mM) over 1 h, with and without previous treatment for 2 h with rutin-nanoliposomes (phospholipid 25 μ M + rutin 16.7 μ M). The fluorescence (λ_{exc} = 485 nm; λ_{em} = 535 nm) was determined in a microplate fluorimeter (SynergyTM H1, Biotek Instruments Winooski, USA) and compared with the respective controls. Results were expressed following the equation: *Intracellular reactive species (%)* = $\frac{Abs\ sample}{Abs\ control} \times 100$.

3.5.4.2. Assays with BV-2 cell line

3.5.4.2.1. Cell culture conditions

The murine microglia BV-2 cell line was routinely cultured, and maintained, with the culture medium DMEM+GlutaMAX (supplemented with 10% FBS and 1% Pen Strep). Cells were kept in a controlled humidified atmosphere of 5% CO₂, at 37 °C, in 25 cm³ culture flasks. When cells reached a near-confluent stage, the monolayer was detached by scrapping, collected to a sterile conical centrifuge tube, and diluted in culture medium (6 mL). To determine cell density/mL, cells were manually quantified in a Neubauer chamber with the trypan-blue exclusion test.

3.5.4.2.2. Cell viability

Cell viability was assessed in terms of MTT reduction by mitochondrial dehydrogenases (**Figure 32**). Serial dilutions of *T. catigua* and *T. diffusa* crude extracts, *T. catigua* and *T. diffusa* nanophytosomes, and blank nanoliposomes, were all prepared with culture medium and sterilized by passing through a 0.22 μ m pore size membrane. BV-2 cells were seeded at 40000 cells/well in 96-wells plates and incubated for 24 h. After this period, the culture medium was discarded and replaced by 0.5 mg/mL MTT solution (prepared in culture medium) and cells were incubated for 1 h. Finally, the culture medium was removed from each well and formazan crystals solubilized in DMSO:isopropanol (3:1, v/v). The absorbance was determined at 510 nm in a plate reader and compared to that from respective controls. Results were expressed following the equation: *Cell viability (%)* = $\frac{Abs\ sample}{Abs\ control} \times 100$.

3.5.4.2.3. Insult with IFN- γ and quantification of nitrite

BV-2 cells were seeded in DMEM+GlutaMAX at 40 000 cells/well in 96-wells plates. The production of NO, evaluated in terms of nitrite accumulated in the culture supernatant, was assessed in non-stimulated BV-2 cells (control) and cells stimulated for 21 h with IFN- γ (20 U/mL).

To evaluate the protective effects of the test samples against the pro-inflammatory stimulus, BV-2 cells were also pre-treated with *T. catigua* and *T. diffusa* crude extracts, blank nanoliposomes, and *T. catigua* and *T. diffusa* nanophytosomes for 3 h, followed by a 21 h period of co-incubation with IFN- γ . After that, 100 μ L of culture medium were collected from each well and mixed with an equal volume of Griess reagent. The absorbance was measured at 562 nm in a spectrophotometer microplate reader and compared to that from respective controls. Results were expressed following the equation: *NO levels* (%) = $\frac{Abs\ sample}{Abs\ control} \times 100$. Finally, the cell viability was assessed in terms of MTT reduction, as detailed above.

3.6. Data and statistics

Phenolic compounds were quantified from three determinations of three independent extractions and expressed as mean $\pm$ SD. Three SPE procedures were performed for each plant material. The PL-rich fractions were obtained from four independent extractions, each one starting from two egg yolks of different egg batches. Three aliquots of every PL-rich fraction were collected for characterization and the results expressed as mean $\pm$ SD. Limit of quantification (LOQ) and limit of detection (LOD) were calculated from the residual standard deviation (σ) of the regression equation and the slope (S), according with the following equations: $LOD = 3\ \sigma/S$; $LOQ = 10\ \sigma/S$. The entrapment efficiency, Zeta potential and particle size were determined from three independent formulations. IC₅₀ and IC₂₅ values were calculated from three independent experiments, each one performed in triplicate, and results expressed as mean $\pm$ SEM. Michaelis-Menten kinetic parameters, *K_m* (Michaelis constant) and *V_{max}* (maximal velocity), curve fittings and statistical variations were calculated with GraphPad Prism 8.2 software (San Diego, CA, USA). At least five experiments, each one performed in triplicate, were conducted for cellular assays and the results were expressed as mean $\pm$ SEM. One way ANOVA followed by Tukey's *post hoc* test was applied (level of statistical significance $p < 0.05$) with the same software.

CHAPTER III

Results and discussion

4. Results and discussion

4.1. Phenolic profile of the crude extracts

4.1.1. Phenolic profile of *T. catigua* stem bark aqueous extract

HPLC-DAD-ESI/MSⁿ and UPLC-ESI-QTOF-MS analysis revealed the presence of seven quinic acid derivatives (compounds **1–3**, **5–7** and **25**), three flavan-3-ols (**4**, **8** and **10**) and at least sixteen flavan-3-ols derivatives (**9**, **11–24** and **26**) (**Figure 33**).

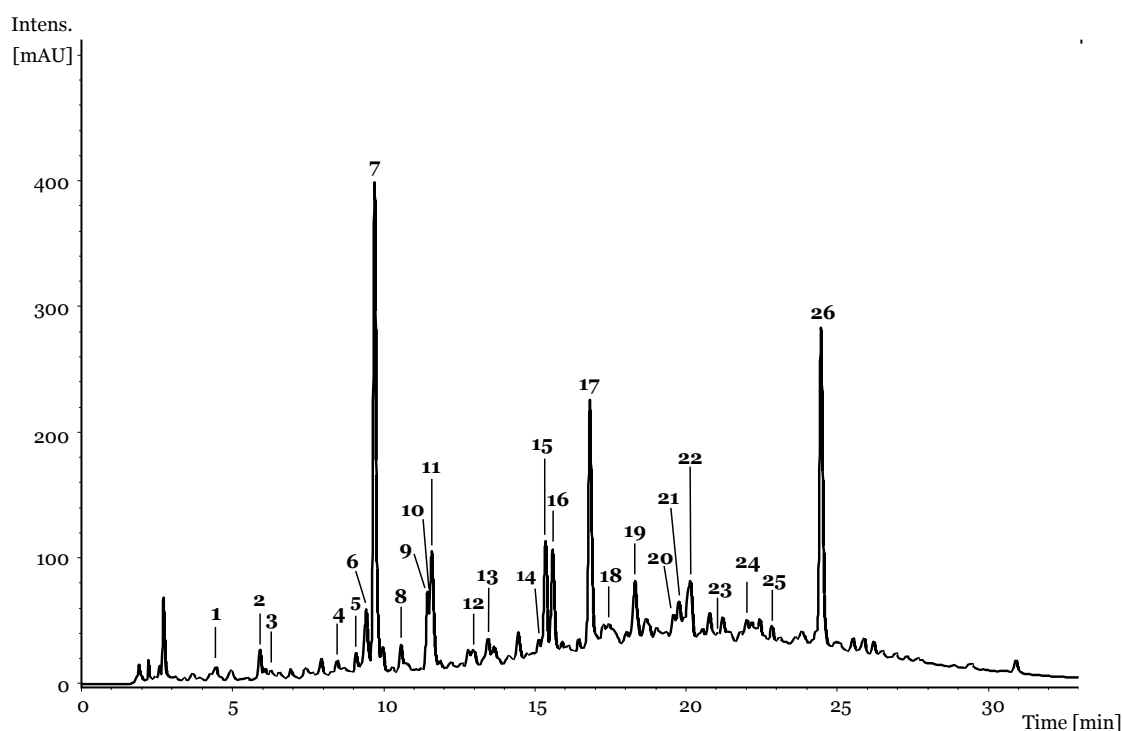


Figure 33. HPLC-DAD chromatogram (at 280 nm) of *T. catigua* crude aqueous extract. **1:** 3-*O*-Methoxybenzoylquinic acid; **2:** 3-*O*-Caffeoylquinic acid; **3:** 4-*O*-Methoxybenzoylquinic acid; **4:** Catechin; **5:** 5-*O*-Methoxybenzoylquinic acid; **6:** 4-*O*-Caffeoylquinic acid; **7:** 5-*O*-Caffeoylquinic acid; **8:** Procyanidin; **9:** Dihydroapocynin E type; **10:** Epicatechin; **11:** Dihydroapocynin E type; **12:** Apocynin E type; **13:** Cinchonain II type; **14:** Apocynin E type; **15:** Cinchonain IIa; **16:** Cinchonain IIb; **17:** Cinchonain Ia; **18:** Apocynin E type; **19:** Cinchonain I type; **20:** Cinchonain I type; **21:** Cinchonain I type; **22:** Cinchonain I type; **23:** Apocynin E type; **24:** Cinchonain I type; **25:** 4,5-di-*O*-Caffeoylquinic acid; **26:** Cinchonain Ib.

Table 6. Rt, UV, molecular formula, MSⁿ [M-H]⁻ and content of the phenolic compounds identified in *T. catigua* stem bark crude aqueous extract^a.

Compounds ^b	Rt (min)	UV (nm)	Molecular formula	[M-H] ⁻ , <i>m/z</i>	MS ² [M-H] ⁻ , <i>m/z</i> (%)	mg.g ⁻¹ lyophilized ^c
Quinic acid derivatives						
1	4.4	276	C ₁₅ H ₁₈ O ₈	325.0935	191(20), 151(100)	0.35 ± 0.02
3	6.3	276	C ₁₅ H ₁₈ O ₈	325.0937	173(100), 151(10)	0.54 ± 0.05
5	8.3	276	C ₁₅ H ₁₈ O ₈	325.0936	191(100)	0.01 ± 0.00
2	5.9	295sh, 325	C ₁₆ H ₁₈ O ₉	353.0904	191(100), 179(55)	3.47 ± 0.10
6	9.4	296sh, 326	C ₁₆ H ₁₈ O ₉	353.0906	179(55), 173(100)	3.05 ± 0.48
7	9.7	297sh, 326	C ₁₆ H ₁₈ O ₉	353.0899	191(100)	38.26 ± 1.20
25	22.8	296sh, 328	C ₂₅ H ₂₄ O ₁₂	515.1202	353(100) ^d	1.19 ± 0.16
Flavan-3-ols						
4	8.5	280	C ₁₅ H ₁₄ O ₆	289.0747	245(100), 205(30), 179(40)	1.48 ± 0.21
8	10.6	280	C ₃₀ H ₂₆ O ₁₂	577.1353	425(100), 407(65), 289(20)	3.42 ± 0.32
10	11.5	---	C ₁₅ H ₁₄ O ₆	289.0747	245(100), 205(40), 179(20)	45.60 ± 1.57 ^e
Phenylpropanoid substituted flavan-3-ols						
9	11.4	280	C ₂₄ H ₂₂ O ₁₀	469.1140	315(15), 289(100), 245(10)	22.13 ± 1.42
11	11.6	280	C ₂₄ H ₂₂ O ₁₀	469.1141	315(10), 289(100), 245(5)	45.60 ± 1.57 ^e
12	13.0	280	C ₂₄ H ₂₀ O ₁₀	467.0997	357(100), 327(30), 217(25)	4.97 ± 0.73
14	15.2	282	C ₂₄ H ₂₀ O ₁₀	467.0994	398(17), 357(100), 217(20)	0.44 ± 0.00
18	17.4	281	C ₂₄ H ₂₀ O ₁₀	467.0997	357(100), 217(60)	2.52 ± 0.02
23	21.0	---	C ₂₄ H ₂₀ O ₁₀	467.0997	357(100), 217(20)	1.02 ± 0.11
13	13.5	280	C ₃₉ H ₃₂ O ₁₅	739.1670	587(100), 449(30), 289(40)	12.37 ± 1.71
15	15.3	280	C ₃₉ H ₃₂ O ₁₅	739.1669	587(100), 449(60), 289(30)	45.23 ± 4.43
16	15.6	280	C ₃₉ H ₃₂ O ₁₅	739.1668	587(100), 449(30), 289(25)	44.41 ± 5.10
17	16.8	280	C ₂₄ H ₂₀ O ₉	451.1048	341(100)	105.24 ± 10.44
19	18.3	282	C ₂₄ H ₂₀ O ₉	451.1052	341(100), 299(95)	11.40 ± 1.22
20	19.6	282	C ₂₄ H ₂₀ O ₉	451.1050	341(50), 299(100)	2.73 ± 0.20
21	19.8	282	C ₂₄ H ₂₀ O ₉	451.1051	341(100), 299(5)	17.93 ± 1.27
22	20.1	282	C ₂₄ H ₂₀ O ₉	451.1050	341(100)	14.61 ± 0.12
24	19.8	282	C ₂₄ H ₂₀ O ₉	451.1053	341(100), 299(3)	6.10 ± 0.09
26	24.4	281	C ₂₄ H ₂₀ O ₉	451.1052	341(100)	126.56 ± 6.46
						Σ = 551.02 ± 37.61

^a Main observed fragments. ^b Identity as in **Figure 33**. ^c Results expressed as mean ± SD. ^d **25** MS³(515 → 353): 191(20), 179(80) 173(100) and absence of ion at *m/z* 335. ^e Compounds **10** and **11** quantified together.

In **Table 6**, the identified compounds are grouped by classes (quinic acid derivatives, flavan-3-ols and procyanidins, and phenylpropanoid substituted flavan-3-ols). Within each class, the several compounds were grouped according to their structural similarity (methoxybenzoyl quinic acids, caffeoylquinic acids, apocynin E type, cinchonain II type, cinchonain I type), following their elution order.

4.1.1.1. Quinic acid derivatives

Compound **7** is the peak with the highest absorbance at 280 nm (**Figure 33**). Its UV spectrum, characteristic of a cinnamoyl acid derivative, along with its deprotonated molecular ion and MS fragmentation (**Table 6**) allow its identification as 5-*O*-caffeoylquinic acid (neochlorogenic acid) (403).

With identical UV spectra and mass, only differing in mass fragmentation (**Table 6**), compounds **2** and **6** are identified as 3-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid, respectively. Compound **25**, with the same UV spectrum and showing the $[M-H]^-$ at m/z 515.1202, is a di-caffeoylquinic acid, and its MS fragmentation corresponds to that of 4,5-di-*O*-caffeoylquinic acid (383).

The other quinic acid esters (**1**, **3** and **5**) showed UV spectra with a maximum at 276 nm and deprotonated molecular ions at m/z 325.0936. In their MS fragmentation are observed the ions characteristic of quinic acid, (i.e., 191 $[\text{quinic acid-H}]^-$ (**1** and **5**) and 173 $[(\text{quinic acid-H})-18]^-$ (**3**)), as well as the ion at m/z 151 $[\text{methoxybenzoic acid-H}]^-$ (**1** and **3**). In addition, their cleavage patterns were similar to that observed for the caffeoylquinic acids referred above. Therefore, they are identified as 3-*O*-methoxybenzoylquinic acid (**1**), 4-*O*-methoxybenzoylquinic acid (**3**) and 5-*O*-methoxybenzoylquinic acid (**5**).

From all these compounds, only chlorogenic acid (**7**) was previously reported in *T. catigua* stem bark extracts (336,384,385).

4.1.1.2. Flavan-3-ols and oligomers

Some other compounds present UV spectra with a maximum of absorbance at ~280 nm and deprotonated molecular ion of flavan-3-ols (monomers **4** and **10**, and dimer **8**) (**Table 6**). Compounds **4** and **10**, with molecular mass of catechin/epicatechin ($[M-H]^-$, m/z 289.0747), were already described in this species (**Figure 13**) (336,383,385). In

reversed-phase chromatography, catechin elutes first than epicatechin, and they are coincident with **4** and **10**, respectively.

Two dimers have been previously reported in *T. catigua*: procyanidin B2 [epicatechin-(4→8)-epicatechin] and procyanidin B4 [catechin-(4→8)-epicatechin (**Figure 15**). In this extract it was detected the presence of one compound (**8**) with deprotonated molecular ion at m/z 577.1353 ($C_{30}H_{26}O_{12}$) (**Table 6**) that may correspond to one of them. Another compound, found in trace amounts and with the same mass, co-eluted with compound **7** (not indicated in **Figure 33** and **Table 6**) and may correspond to the other procyanidin. It was not possible to detect procyanidin C1 [epicatechin-(4→8)-epicatechin-(4→8)-epicatechin], a trimer described before in this species (336).

4.1.1.3. Phenylpropanoid substituted flavan-3-ols

Other four compounds are detected in reduced or trace amounts (**12**, **14**, **18** and **23**) (**Figure 33**) with deprotonated molecular ions at m/z 467.0997 (**Table 6**), which may be phenylpropanoid substituted epigallocatechins ($C_{24}H_{20}O_{10}$). Given that apocynin E was already identified in *T. catigua* stem bark (336), and it must correspond to one of these compounds, in this work we considered the four compounds as apocynins E type. Compounds **9** and **11** presented 2 amu higher masses (m/z 469.1140) and molecular formula $C_{24}H_{22}O_{10}$. Therefore, they were tentatively identified as being from dihydroapocynin E type. As far as we know, this is the first report of these compounds in this species.

Seven compounds (**17**, **19–22**, **24** and **26**) present the molecular formula $C_{24}H_{20}O_9$ and their mass ($[M-H]^-$, m/z 451.105) was 16 amu lower than that of **12**, **14**, **18** and **23**, thus corresponding to phenylpropanoid substituted epicatechins. In *T. catigua* stem bark were already described epicatechins with additional phenylpropanoid (C_6-C_3) units with substitutions positioned at C8, cinchonains Ia and Ib (**Figure 14**) (338–341,384), and at C6, cinchonains Ic and Id (**Figure 14**) (339), which have the same mass. As so, these compounds were generically labelled as being from cinchonain I type. Cinchonains Ia and Ib are the most common in *T. catigua* bark and may correspond to the most abundant ones (**17** and **26**, respectively). Catiguanins A and B ($C_{25}H_{22}O_{10}$), two other phenylpropanoid substituted epicatechins (339), are not detected in this extract.

In this extract are also detected procyanidins with a phenylpropanoid substitution and deprotonated molecular ion at m/z 739.1669 ($C_{39}H_{32}O_{15}$) (compounds **13**, **15** and **16**)

(**Table 6**). Two proanthocyanidin dimers substituted with a phenylpropanoid moiety at the A-ring and with this mass were previously reported in *T. catigua* stem bark: cinchonain IIa and cinchonain IIb (**Figure 15**) (338,341,384). Thus, these compounds were generically named cinchonain II type, with the most abundant ones (**15** and **16**) probably coinciding with cinchonain IIa and cinchonain IIb, respectively (**Table 6**).

Other phenolic compounds described before in this material, namely gallic, ellagic, caffeic and rosmarinic acids (**Figure 12**), and the flavonols quercetin, kaempferol, rutin, quercitrin and isoquercitrin (**Figure 13**) are not detected in this aqueous extract.

4.1.1.4. Quantitative analysis of the identified phenolic compounds

The quantified phenolic fraction of *T. catigua* stem bark crude aqueous extract corresponds to 551.02 ± 37.61 mg/g of lyophilized extract (**Table 6**). This extract is richer in flavonoids, namely flavan-3-ols, oligomers and phenylpropanoid derivatives, than in quinic acid derivatives. Particularly, the phenylpropanoid substituted chemical subclass is the dominant one, accounting for 417.64 mg/g of the lyophilized extract, which represents ~81% of the total quantified mass (**Table 6**). Compounds **26** and **17**, two cinchonain I type compounds, are the most abundant ones (respectively, 126.56 ± 6.46 and 105.24 ± 10.44 mg/g of lyophilized extract). Among quinic acid esters, 5-*O*-caffeoylquinic acid (**7**) is the major compound (38.26 ± 1.20 mg/g of lyophilized extract).

From a quantitative point of view, the comparison of these results with the available literature is difficult, owing to the distinct nature of the extracting solvents and procedures. For instance, some authors found a considerably lower number of quantified polyphenols in *T. catigua* bark aqueous extract (85.12 ± 5.57 mg/g of dry weight extract), possibly explained by the reduced number of identified/quantified compounds (386).

4.1.2. Phenolic profile of *T. diffusa* leave aqueous extract

The HPLC-DAD-ESI/MSⁿ and UPLC-ESI-QTOF-MS analysis reveal a complex and rich mixture of phenolic compounds (**Figure 34**). Regarding the less representative compounds, with UV spectra characteristic of cinnamoyl acid derivatives (~295, 325 nm), their specific MS fragmentation patterns could not be fully elucidated.

Nevertheless, thirty-seven compounds with UV spectra of flavonoid derivatives ($R_t > 7$ min) are identified. Their MS fragmentation allows to generally classify them as aglycones, C-glycosides, O-glycosides and cinnamoyl derivatives. The conjugated sugar moieties are most of the times hexoses and deoxyhexoses.

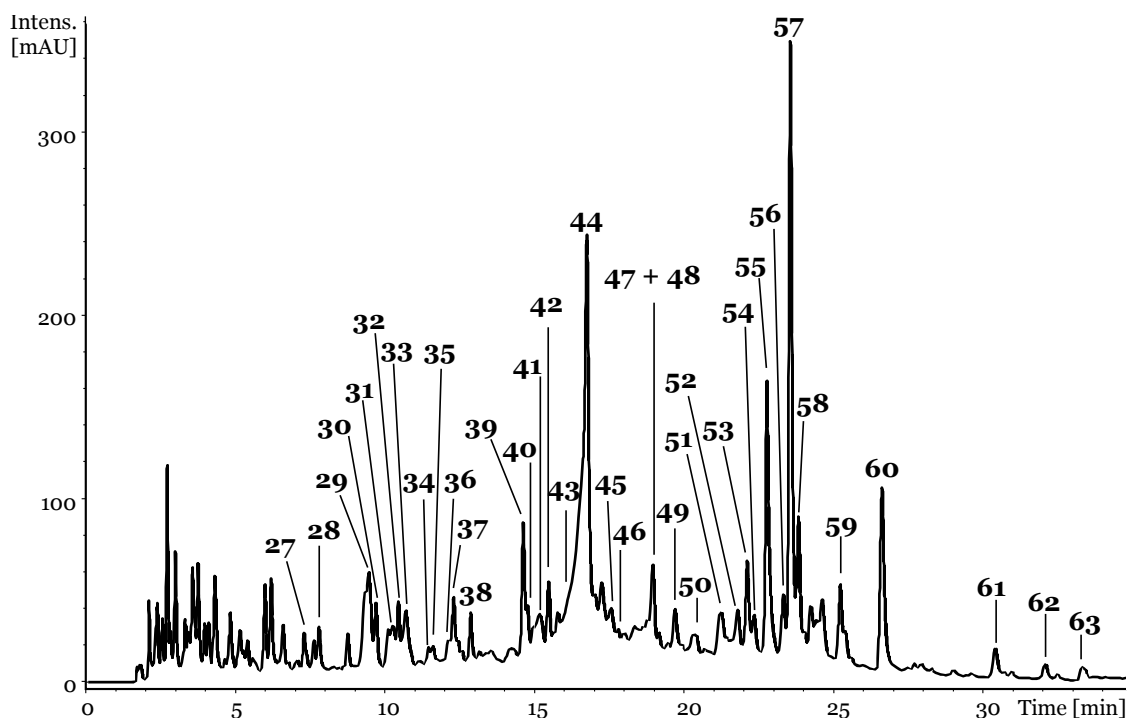


Figure 34. HPLC-DAD chromatogram (340 nm) of *T. diffusa* crude aqueous extract. **27**: Apigenin 6,8-di-C-glucoside; **28**: Myricetin-3-O-(6-glucosyl)glucoside; **29**: Luteolin-8-C-(2-rhamnosyl)glucoside; **30**: Quercetin-3-O-(6-glucosyl)glucoside; **31**: Apigenin-8-C-(2-glucosyl)glucoside; **32**: Larycitrin-3-O-(6-glucosyl)glucoside; **33**: Apigenin-8-C-(2-rhamnosyl)glucoside; **34**: Acacetin-6,8-di-C-glucoside; **35**: Quercetin-3-O-(6-rhamnosyl)glucoside; **36**: Quercetin-3-O-glucoside; **37**: Isorhamnetin-3-O-(6-glucosyl)glucoside; **38**: Syringetin-3-O-(6-glucosyl)glucoside; **39**: Apigenin-7-O-glucoside; **40**: Quercetin-3-O-(2-rhamnosyl)ketodeoxihexoside; **41**: Luteolin-8-C-(2-rhamnosyl)quinovoside; **42**: Luteolin-6-C-(2-ketodeoxihexosyl)hexoside; **43**: Luteolin-8-C-(2-ketodeoxihexosyl)hexoside; **44**: Luteolin-8-C-(2-rhamnosyl)ketodeoxihexoside; **45**: Luteolin-8-C-(2-deoxihexosyl)ketohehexoside; **46**: Luteolin-6-C-(2-rhamnosyl)ketodeoxihexoside; **47**: Apigenin-7-O-(2-rhamnosyl)ketodeoxihexoside; **48**: Luteolin-6-C-(2-deoxihexosyl)ketohehexoside; **49**: Luteolin; **50–52**: Luteolin derivatives; **53**: Luteolin-8-C-*E*-propenoic acid; **54**: Luteolin acylated derivative; **55**: Apigenin-7-O-(4"-*p*-*E*-coumaroyl)glucoside; **56**: Apigenin; **57**: Apigenin-7-O-(6"-*p*-*E*-coumaroyl)glucoside; **58**: Apigenin-7-O-(6"-*p*-*Z*-coumaroyl)glucoside; **59**: Apigenin-7-O-(4"-*p*-*Z*-coumaroyl)glucoside; **60**: Apigenin-8-C-propionic acid; **61**: Dihydroxyflavone-8-C-propionic acid; **62**: Acacetin; **63**: Velutin.

4.1.2.1. Flavonoid-di-C-glycosides

Compounds **27** and **34**, flavonoids with a single B-ring substitution, show MS fragments of C-hexosides (-90/120). Their typical molecular ions of di-C-hexosyl flavonoid derivatives (AGly+113/AGly+83) are related to apigenin in **27** (5,7,4'-trihydroxyflavone), and to a dihydroxy-methoxyflavone in **34** (Table 7). Another dihydroxy-methoxyflavone aglycone is detected in compound **62**, this one in traceable amounts, being previously identified in this species as acacetin (5,7-dihydroxy-4'-methoxyflavone) (Figure 18). Therefore,

compound **27** is tentatively identified as vicenin-2 (apigenin-6,8-di-*C*-glucoside), the most abundant apigenin 6,8-di-*C*-hexosyl derivative in Nature (387). Compound **34**, a di-*C*-hexosyl dihydroxy-methoxyflavone, is tentatively identified as acacetin-6,8-di-*C*-glucoside. These compounds were herein described in this species for the first time.

RESULTS AND DISCUSSION

Table 7. Rt, UV, molecular formula (M), MSⁿ [M-H]⁻ data of the flavonoid glycosides identified in the crude *T. diffusa* crude aqueous extract.^a

Compound ^b	Rt (min)	UV (nm)	M	[M-H] ⁻ , <i>m/z</i>	MS ² [M-H] ⁻ , <i>m/z</i> (%)							
Flavonoid-di-C-glycosides												
					<u>-90</u>	<u>-120</u>		<u>[AGly+113]⁻</u>	<u>[AGly+83]⁻</u>			
27	7.3	270, 334	C ₂₇ H ₃₀ O ₁₅	593.1490	503(35)	473(90)		383(80)	353(100)			
34	11.4	274, 328 ^c	C ₂₈ H ₃₂ O ₁₅	607.1668	517(50)	487(100)		397(35)	367(95)			
Mono-C-glycosyl-O-glycosylated flavonoids												
					<u>-104</u>	<u>-118</u>	<u>-120</u>	<u>-164</u>	<u>-180</u>	<u>[AGly+71]⁻</u>	<u>[AGly+41]⁻</u>	<u>[AGly+41-18]⁻</u>
29	9.5	255, 266sh, 348	C ₂₇ H ₃₀ O ₁₅	593.1490			473(50)	429(40)		357(45)	327(100)	
31	10.3	—	C ₂₇ H ₃₀ O ₁₅	593.1490			473(12)		413(100)			293(50)
33	10.7	268, 332	C ₂₇ H ₃₀ O ₁₄	577.1563			457(5)	413(100)				293(45)
41	15.1	256, 266, 292sh, 348	C ₂₇ H ₃₀ O ₁₄	577.1563	473(35)			413(100)		357(55)	327(100)	
42	15.4	254, 266, 298sh, 348	C ₂₇ H ₂₈ O ₁₅	591.1345			471(70)				327(100)	
43	16.0	—	C ₂₇ H ₂₈ O ₁₅	591.1345 ^d			471(50)				327(50)	
44	16.6	254, 266, 298sh, 348	C ₂₇ H ₂₈ O ₁₄	575.1369 ^e				411(100)				
45	17.5	—	C ₂₇ H ₂₈ O ₁₅	591.1363 ^f		473(30)						
46	17.7	—	C ₂₇ H ₂₈ O ₁₅	591.1370		473(80)					327(100)	
48	18.9	268, 340 ^c	C ₂₇ H ₂₈ O ₁₅	591.1348		473(100)		427(6)			327(70)	

Table 7. *Cont.*

Compound ^b	Rt (min)	UV (nm)	M	[M-H] ⁻ , m/z	MS ² [M-H] ⁻ , m/z (%)	
Flavonoid- <i>O</i> -glycosides						
					<u>-164</u>	<u>[AGly-H]⁻</u>
28	7.8	255, 266sh, 305sh, 355	C ₂₇ H ₃₀ O ₁₈	641.1312		316(100)
30	9.7	255, 266sh, 302sh, 355	C ₂₇ H ₃₀ O ₁₇	625.1312		301(100)
32	10.4	254, 266sh, 306sh, 358	C ₂₈ H ₃₂ O ₁₈	655.1485		331(100)
35	11.6	255, 266sh, 301sh, 354 ^c	C ₂₇ H ₃₀ O ₁₆	609.1461		301(100)
36	12.1	—	C ₂₁ H ₂₀ O ₁₂	463.0869		301(100)
37	12.3	254, 266sh, 298sh, 354	C ₂₈ H ₃₂ O ₁₇	639.1567		315(100)
38	12.9	254, 266sh, 302sh, 358	C ₂₉ H ₃₄ O ₁₈	699.1672		345(100)
39	14.6	266, 336	C ₂₁ H ₂₀ O ₁₀	431.0962		269(100)
40	14.7	—	C ₂₇ H ₂₈ O ₁₅	591.1355 ^g	427(50)	301(100)
47	18.8	268, 340 ^c	C ₂₇ H ₂₈ O ₁₃	559.1431 ^h	395(100)	269(15)

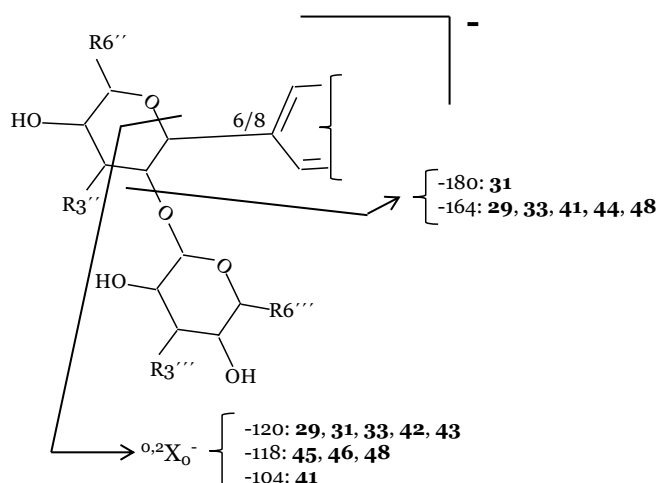
Table 7. *Cont.*

Compound ^b	Rt (min)	UV (nm)	M	[M-H] ⁻ , m/z	MS ² [M-H] ⁻ , m/z (%)	
					Flavonoid- <i>O</i> -glycoside cinnamoyl derivatives	
					-146	[AGly-H] ⁻
55	22.8	268, 320	C ₃₀ H ₂₆ O ₁₂	577.1317	431(10)	269(100)
57	23.5	268, 318	C ₃₀ H ₂₆ O ₁₂	577.1320	431(1)	269(100)
58	23.8	268, 320	C ₃₀ H ₂₆ O ₁₂	577.1320	431(1)	269(100)
59	25.2	268, 318	C ₃₀ H ₂₆ O ₁₂	577.1320	431(30) 413(18)	269(100)

^a Main observed fragments.^b Identity as in **Figure 34**.^c The co-elution with other compounds hinders the correct acquisition of its UV spectrum.^d **43** MS²(591): 575(100, -16), 471(50), 327(50).^e **44** MS²(575): 411(100, -164), 301(90), 285(30); MS³(411): 301(100), 285(40).^f **45** MS²(591): 547(30, -44), 473(30, -118), 383(100), 311(35); MS³(383): 311(100).^g **40** MS³(427): 301(100).^h **47** MS²(559): 395(100), 351(10), 321(20), 269(15); MS³(395): 351(30), 321(100), 269(60).

4.1.2.2. Mono-C-glycosyl-O-glycosylated flavonoids

In this aqueous extract ten flavonoid-mono-C-glycosides were identified (compounds **29**, **31**, **33**, **41–46** and **48**) with an additional O-glycosylation. The MS fragmentation of **29**, **33** and **41** show extensive losses of -164 amu (146+18), and of -180 amu (162+18) in compound **31**. Those are indicative of O-deoxihexosylation and O-hexosylation, respectively, over a non-phenolic hydroxyl group (388). Compounds **29**, **31** and **33** presented cleavages of -120 amu that, just like discussed above, indicate the presence of C-glycosylation with a hexose moiety. As for compound **41**, the loss of 104 amu fragment suggests a C-deoxihexosyl substitution (**Table 7**) (389). In addition, the loss of these fragments (-120/-104) results from the internal cleavage of the sugar moiety in O,2 positions of the C-glycosylation (which contains the carbons 6''–3''), to form the $^{0,2}X_o^-$ ion. This event places the O-glycosylation at 2'' (**Figure 35**).



Compounds		R3''	R6''	R3'''	R6'''
29	Luteolin-8-C-(2-rhamnosyl)glucoside	OH	CH ₂ OH	OH	CH ₃
31	Apigenin-8-C-(2-glucosyl)glucoside	OH	CH ₂ OH	OH	CH ₂ OH
33	Apigenin-8-C-(2-rhamnosyl)glucoside	OH	CH ₂ OH	OH	CH ₃
41	Luteolin-8-C-(2-rhamnosyl)quinovoside	OH	CH ₃	OH	CH ₃
42	Luteolin-6-C-(2-ketodeoxihexosyl)hexoside	OH	CH ₂ OH	O=	CH ₃
43	Luteolin-8-C-(2-ketodeoxihexosyl)hexoside	OH	CH ₂ OH	O=	CH ₃
44	Luteolin-8-C-(2-rhamnosyl)ketodeoxihexoside	O=	CH ₃	OH	CH ₃
45	Luteolin-8-C-(2-deoxihexosyl)ketohehexoside	O=	CH ₂ OH	OH	CH ₃
46	Luteolin-6-C-(2-rhamnosyl)ketodeoxihexoside	O=	CH ₂ OH	OH	CH ₃
48	Luteolin-6-C-(2-deoxihexosyl)ketohehexoside	O=	CH ₂ OH	OH	CH ₃

Figure 35. General fragmentation patterns of the identified mono-C-glycosyl-O-glycosylated flavonoids in the *T. diffusa* crude aqueous extract.

The aglycone of **29** and **41** is identified as luteolin due to the presence of their characteristic ions (AGly+71/AGly+41) of mono-C-glycosyl flavones (357/327, 286+71/286+41: luteolin) (387). In the O-glycosyl-C-glycosyl flavones **31** and **33**, some ions with loss of water are observed (m/z 293, AGly+41-18, 270+41-18: apigenin) (Table 7) (388). Altogether, compound **29** can be labelled as luteolin-C-(2-deoxihexosyl)hexoside and compound **41** as luteolin-C-(2-deoxihexosyl)deoxihexoside. Previous works have reported the presence of luteolin-8-C-(2-rhamnosyl)glucoside and luteolin-8-C-(2-rhamnosyl)quinovoside in this species, matching with **29** and **41**, respectively (390,391). The same authors identified apigenin-8-C-(2-rhamnosyl)glucoside, which matches with compound **33** (391). Apigenin-8-C-(2-glucosyl)glucoside (**31**) is here identified for the first time in *T. diffusa*.

Compounds **42–46** and **48** are C-glycosyl-O-glycosides with some keto-glycosides in their structures. The MS fragmentations of these molecules do not always present similar patterns, or even specific molecular ions (388,389). For the compounds herein detected it was not possible to establish a general fragmentation scheme.

One of the major compounds in the extract (**44**) presented UV spectrum of a luteolin derivative, with $[M-H]^-$ ion at m/z 575.1369. In its MS² was observed a base peak at m/z 411 ($[(M-H)-164]^-$) from a deoxihexosylation substitution over a non-phenolic hydroxyl. Other detected ions, such as those at m/z 301 and 285, point to a O-glycosylated derivative of either luteolin or quercetin. However, the acid hydrolysis of the native extract did not result in quercetin, neither abundant luteolin. Instead, after treatment, the most abundant compound obtained is a luteolin derivative with $[M-H]^-$ ion at m/z 429. This may be the result of the loss of rhamnose during the partial hydrolysis of compound **44** ($429 = 575 - 146$), and in which MS² fragmentation can still be detected the ion at m/z 301 and the base peak at m/z 338. As so, this compound must be a luteolin C-glycoside with a sugar with 2 amu less than rhamnose ($429 = 285 + 144$), since its MS fragmentation does not show the losses characteristic of C-glycoside, neither the typical ions of the aglycone (388,389,392). A luteolin-C-glycoside with rhamnose placed at 2'', with the same mass of compound **44** and a keto-glycoside with 2 amu less than rhamnose ($575: 285 + 144 + 146$) was already reported in this species (390). The authors had identified it as luteolin-8-C-[6-deoxy-2-O-(α -L-rhamnopyranosyl)-xylo-hexopyranos-3-uloside]. Herein, it will shortly be referred to as luteolin-8-C-(2-rhamnosyl)ketodeoxihexoside.

The MS fragmentation of compounds **42** and **43** ($[(M-H)-]$: 591.1345), other two keto-derivatives, show an internal cleavage of a C-hexoside (-120) and the ion AGly+41 of a mono-C-glycosyl luteolin (Table 7), but not the cleavage of a ketodeoxyhexose at the O-

glycosylation (591= 285+162+144). Considering that it is placed at 2'', these compounds are tentatively identified as luteolin-*C*-(2-ketodeoxyhexoyl)hexoside isomers.

Compounds **45**, **46** and **48** exhibit the same deprotonated molecular ion as compounds **42** and **43**. In their MS fragmentations was observed an abundant loss of 118 amu fragments, similar to that of 120 amu of the *C*-hexosyl derivatives, which most correspond to the internal fragmentation at the 0,2 positions of the ketohexoside (**Figure 35**). Ions at m/z 327, which characterize the main structures of (**42**) and (**43**) as luteolin mono-*C*-glycosyl derivatives, were also observed for (**46**) and (**48**), but not for compound **45** (**Table 7**). Since the *O*-deoxyhexosyl cleavage (-164=146+18) of compound **48** also originates a low abundant ion at m/z 427, these must be luteolin-*C*-(2-deoxyhexosyl)ketohexoside isomers. The relative abundance of the ions [(M-H)-120/-118] and [AGly+41] is higher in compounds **42**, **46**, and **48**, than in **43** and **45**, and the ion corresponding to AGly+41 is absent in the fragmentation of compound **45**. As so, in the structures of **42**, **46** and **48** the *C*-glycosylation is placed at C6 instead of C8, which is a more preferential cleavage pattern. Except for compound **44**, all the other nine flavonoid *C*-derivatives with keto-glycosides are here identified for the first time in *T. diffusa*.

4.1.2.3. Flavonoid-*O*-glycosides

In this group are included compounds **28**, **30**, **32**, **35**, **37** and **38**, all featuring UV spectra of 3-substituted flavonols with an additional B-ring substitution. Their deprotonated molecular ions indicate a diglycosilated backbone structure. The MS² fragmentations originate base peaks of the correspondent deprotonated aglycones (**Table 7**). It is also observed the absence of the ions derived from breaking the 1→6 interglycosidic linkage, which is hard to break. The presence of gentiobiosides (glucosyl(1→6)glucoside) in *T. diffusa* was already reported, namely derivatives of quercetin (3,5,7,3',4'-pentahydroxyflavone), laricitrin (3,5,7,3',4'-pentahydroxy-5'-methoxyflavone) and syringetin (3,5,7,4'-tetrahydroxy-3',5'-dimethoxyflavone) (**Figure 17**) (390). These compounds are also present in this extract, being identified respectively as compounds **30**, **32** and **38**. As far as we know, the other compounds with gentiobiose are in this species for the first time, namely derivatives of myricetin (3,5,7,3',4',5'-hexahydroxyflavone) (**28**), and isorhamnetin (3,5,7,3'-tetrahydroxy-4'-methoxyflavone) (**36**). Quercetin-3-*O*-rutinoside(rhamnosyl(1→6)glucoside) is also identified (**35**).

The diglycosides **40** and **47** exhibit a loss of 164 amu in their MS² fragmentations, which is a sign of a linkage between a rhamnosyl moiety and a non-phenolic hydroxyl group

(**Table 7**). In the particular case of compound **40**, it is observed an ion at m/z 301 (base peak) corresponding to the deprotonated aglycone (quercetin-H). A ketodeoxyhexose should be the second glycosyl moiety ($591 = 301 + 146 + 144$). In addition, $MS^3 [(591) \rightarrow (591 - 164)]^-$ originates the same base peak ion at m/z 301, thus compound **40** is labelled as quercetin-3-*O*-(2-rhamnosyl)ketodeoxyhexoside. The MS fragmentation of **47** show the loss of 164 amu (m/z 395, base peak) and a little abundant ion, probably from the deprotonated apigenin (m/z 269, 15%). However, the MS^3 fragmentation of the base peak is not typical of a flavonoid-*O*-glycoside (**Table 7**, footnotes). This could be due to the presence of other ketodeoxyhexoside ($559 = 269 + 146 + 144$). Therefore, compound **47** is tentatively identified as apigenin-7-*O*-(2-rhamnosyl)ketodeoxyhexoside. The presence of all these keto-glycoside derivatives is here described for the first time.

Quercetin-3-*O*-glucoside (**36**) is also described for the first time, while apigenin-7-*O*-glucoside (**39**) was previously reported (**Figure 18**) (391).

4.1.2.4. Cinnamoyl derivatives of flavonoid-*O*-glycosides

Compounds **55** and **57–59** exhibit UV spectra characteristic of cinnamoyl derivatives with a broad absorption band at ~ 320 nm. They share the same deprotonated molecular ion at m/z 577.1320, and the base peak of deprotonated apigenin (m/z 269) (**Table 7**). The ion at m/z 431 ($[577 - 146]^-$), due to the loss of a *p*-coumaroyl radical, has relative abundance of 10% and 30% in compounds **55** and **59**, respectively, and of $\sim 1\%$ in **57** and **58** (**Table 7**). The low abundance in the last two compounds indicates that the acylation was placed in position 6 of the hexose, which is a strong link that is very hard to break. Therefore, the isomers apigenin-7-*O*-(6''-*p*-*E*-coumaroyl)glucoside (echinacin, **57**) and apigenin-7-*O*-(6''-*p*-*Z*-coumaroyl)glucoside (*Z*-echinacin, **58**), and the pair apigenin-7-*O*-(4''-*p*-*E*-coumaroyl)glucoside (echinaticin, **55**) and apigenin-7-*O*-(4''-*p*-*Z*-coumaroyl)glucoside (*Z*-echinaticin, **59**), are identified in this extract, as before (390).

4.1.2.5. Aglycones and non-glycosylated derivatives

Free aglycones are also present in this aqueous extract of *T. diffusa* (**Table 8**). Apigenin (5,7,4'-trihydroxyflavone) (**56**), acacetin (5,7-dihydroxy-4'-methoxyflavone) (**62**) and velutin (5,4'-dihydroxy-7,3'-methoxyflavone) (**63**), have already been identified in this species (364,390).

Table 8. Rt, UV, molecular formula (M) and [M-H]⁻ data of the flavonoid aglycones and non-glycosylated derivatives identified in *T. diffusa crude aqueous extract*.^a

	Compound	Rt (min)	UV (nm)	M	[M-H] ⁻ , m/z
49	Luteolin	19.6	255, 266sh, 290sh, 348	C ₁₅ H ₁₀ O ₆	285.0400
50	Luteolin derivative	20.4	255, 266sh, 290sh, 348	C ₂₁ H ₁₆ O ₉	411.0711
51	Luteolin derivative	21.2	255, 266sh, 290sh, 348	C ₂₁ H ₁₆ O ₁₁	443.0850
52	Luteolin derivative	21.7	255, 270, 294sh, 350	C ₂₁ H ₁₆ O ₁₀	427.0653
53	Luteolin-8- <i>C-E</i> -propenoic acid	22.1	---	C ₁₈ H ₁₂ O ₈	355.0445 ^b
54	Luteolin acetylated derivative	22.3	255sh, 266, 290sh, 348	C ₂₀ H ₁₄ O ₁₁	429.0448 ^c
56	Apigenin	23.3	266, 338	C ₁₅ H ₁₀ O ₅	269.0448
60	Apigenin-8- <i>C</i> -propionic acid	26.6	266, 338	C ₁₈ H ₁₀ O ₇	337.0338 ^d
61	Dihydroxyflavavone-8- <i>C</i> -propionic acid	30.4	266, 326	C ₁₈ H ₁₀ O ₆	321.0385 ^e
62	Acacetin	32.1	267, 334	C ₁₅ H ₁₂ O ₅	283.0604
63	Velutin	33.3	252, 266sh, 348	C ₁₇ H ₁₄ O ₆	313.0702

^a Main observed fragments.^b **53** MS²(355): 311(100, -44).^c **54** MS²(429): 385(100, -44); MS³(385): 297(100), 285(65).^d **60** MS²(337): 293(100, -44).^e **61** MS²(321): 293(277, -44).

The deprotonated molecular ion at m/z 355.0445, together with the molecular formula C₁₈H₁₂O₈, is indicative that compound **53** is a non-glycosylated luteolin derivative. However, its co-elution with other molecules hindered the acquisition of its UV spectrum (**Table 8**). Furthermore, in its MS² fragmentation a carboxyl group (-44 amu) is cleaved from the main structure, thus it matches with the previously reported luteolin-8-*C-E*-propenoic acid (**Figure 19**) (390).

Newly identified compounds are luteolin (5,7,3',4'-tetrahydroxyflavone) (**49**), one luteolin acylated derivative (**54**) and three other derivatives (**50–52**) of which identities were not possible to achieve. Compound **60** shows UV spectrum of an apigenin derivative, and its molecular mass corresponds to apigenin-*C*-propionic acid. Compound **61** has 16 amu less than **60**, and UV spectrum distinct from chrysin, thus here identified as dihydroxyflavone-*C*-propionic acid (**Table 8**).

4.1.2.6. Quantitative analysis of the identified phenolic compounds

The compounds identified in *T. diffusa* leave crude aqueous extract accounted for a total of 18.20 ± 2.12 mg/g of lyophilized extract (**Table 9**). Luteolin derivatives are the dominant flavonoids corresponding to ~68% of the total quantified compounds. In regard of the specific glycosylated sub-classes, the most represented was the one of mono-C-glycosyl-O-glycosylated flavonoids (12.86 ± 0.61 mg/g of lyophilized extract, ~71%), followed by flavonoid-O-glycosides (3.31 ± 0.33 mg/g of lyophilized extract, ~18%) and by aglycones and non-glycosylated derivatives (1.23 ± 0.17 mg/g of lyophilized extract, ~7%). The other compounds only represent ~4% of the total quantified mass (**Table 9**).

Table 9. Quantification of the phenolic compounds identified in *T. diffusa* crude aqueous extract.

Compound ^a	mg/g of lyophilized extract ^b
27	0.12 ± 0.01
28	0.32 ± 0.01
29	1.96 ± 0.04
30	0.06 ± 0.02
31	0.55 ± 0.03
32	0.26 ± 0.02
33	0.02 ± 0.01
34	0.16 ± 0.02
35	0.21 ± 0.01
36	0.20 ± 0.05
37	0.14 ± 0.01
38	0.40 ± 0.02
39 + 40	1.7 ± 0.44
41	1.62 ± 0.44
42	0.30 ± 0.08
43 + 44	7.47 ± 0.28
45	0.19 ± 0.12
46	0.04 ± 0.01
47 + 48	0.72 ± 0.06
49	0.03 ± 0.01
50	0.08 ± 0.02

51	0.04 ± 0.01
52	0.14 ± 0.01
53	0.18 ± 0.14
54	0.23 ± 0.06
55	0.14 ± 0.05
56	0.08 ± 0.01
57	0.28 ± 0.05
58	0.09 ± 0.04
59	0.01 ± 0.01
60	0.41 ± 0.02
61	0.01 ± 0.01
62	0.02 ± 0.01
63	0.28 ± 0.01
Σ	18.20 ± 2.12

^a Identity as in **Figure 34**.

^b Results expressed as mean $\pm$ SD from three independent determinations

4.2. Screening the neuroprotective potential of *T. catigua* and *T. diffusa* crude aqueous extracts

Neurodegeneration comprehends a complex non-linear intertwined series of pathological events, which irreversibly compromise the brain function. Hence, it is of interest to explore the multiple bioactivities of non-toxic plant extracts through modulation of several biochemical pathways and cell signalling cascades, to reach an effective multitarget therapeutic strategy for the management of neurodegenerative disorders (152).

Herein is explored the *in vitro* neuroactive potential of *T. catigua* and *T. diffusa* crude aqueous extracts considering common targets and biochemical pathways underpinning neurodegeneration.

4.2.1. Antioxidant activity

Antioxidants, such as flavonoids, modulate the redox state of cells by acting through different mechanisms (e.g., hydrogen atom transfer, single-electron transfer, chelation of transition metals, avoiding the formation of hydroperoxides, by enhancing the activity/expression or avoiding the depletion of the enzymatic antioxidant defence systems), thus they have the potential to avoid or attenuate the oxidative stress associated with neurodegenerative processes (393). Since the antioxidant effect of flavonoids is one of their most well-known cell protective bioactivity, here is explored the *in vitro* the potential antioxidant effect of *T. catigua* and *T. diffusa* crude aqueous extracts by several mechanisms.

The TAC was evaluated in terms of the capacity of each extract to reduce molybdenum (VI) to molybdenum (V) with subsequent formation of complexes. When normalized, and expressed as AAE, the results demonstrate that TAC values are always higher (~2-fold) for whichever concentration of *T. catigua* extract than for *T. diffusa* one (**Figure 36**).

In the DPPH• scavenging assay, both extracts display a concentration-dependent effect, and a complete radical scavenging is obtained at the highest concentrations tested (**Figure 36**). In accordance with TAC results, *T. catigua* extract also displays a stronger antiradical effect than *T. diffusa*, as indicated by their IC₅₀ values of 15.92 ± 2.19 and 59.16 ± 8.11 µg lyophilized extract/mL, respectively.

As discussed before, superoxide radical can be originated from mitochondria or by enzymatic activity (e.g., X/XO pathway). In addition, it can undergo dismutation *via* SOD

to form oxygen and hydrogen peroxide and the last can be further converted to highly reactive hydroxyl free radicals *via* Fenton reaction and further impair cellular function (172). Therefore, the direct scavenging of this ROS may prevent deleterious cell effects.

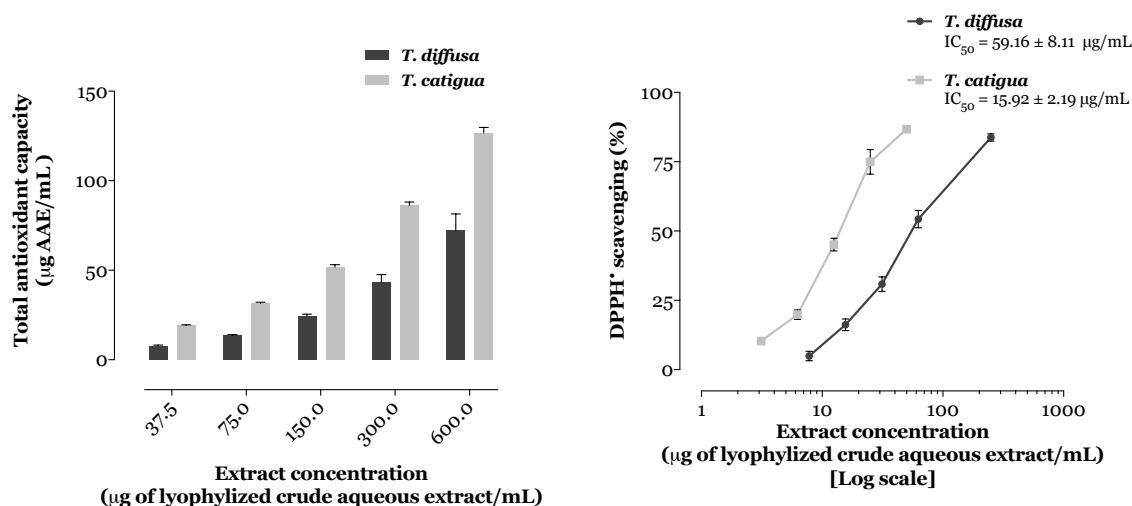


Figure 36. Total antioxidant capacity and DPPH• scavenging activity of *T. diffusa* and *T. catigua* crude aqueous extracts. Results are represented as mean $\pm$ SEM from three independent assays, performed in triplicate. AAE: Ascorbic acid equivalents.

In the *in vitro* superoxide radical scavenging assay, both *T. diffusa* and *T. catigua* crude aqueous extracts display a concentration-dependent effect (**Figure 37**). A complete radical scavenging is verified at the highest concentrations tested, although a more efficient activity is observed for *T. catigua* ($IC_{50} = 11.58 \pm 0.62$ μ g lyophilized extract/mL) than for *T. diffusa* ($IC_{50} = 29.31 \pm 1.65$ μ g lyophilized extract/mL).

As relevant as the direct scavenging is the decrease in superoxide anion radical formation by inhibition of enzymes. For instance, in cytosol, the X/XO pathway is a relevant source of superoxide anion radical, which contributes to oxidative stress and takes part in neurodegenerative processes (394). Therefore, the inhibition of XO activity can result in lower levels of ROS. In the *in vitro* XO inhibition assay it is observed that the crude aqueous extracts of *T. catigua* and *T. diffusa* exhibit a concentration-dependent inhibitory effect (**Figure 37**). Once more, and despite that both extracts cause ~80% enzyme inhibition at the highest concentration tested, the effect of *T. catigua* ($IC_{50} = 22.03 \pm 1.48$ μ g lyophilized extract/mL) is twice higher than that of *T. diffusa* ($IC_{50} = 53.00 \pm 8.89$ μ g lyophilized extract/mL).

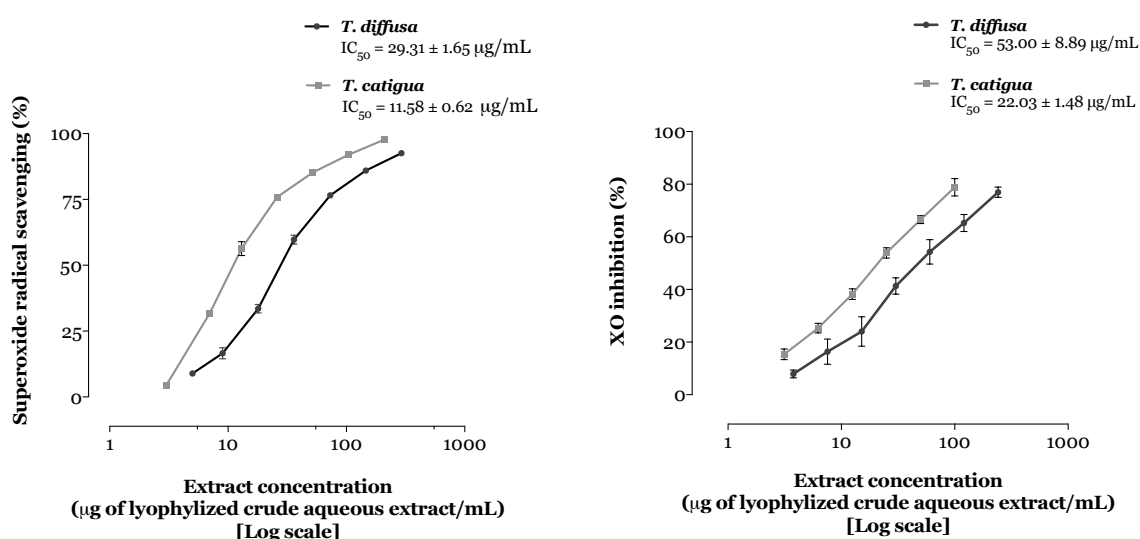


Figure 37. Superoxide radical scavenging and xanthine oxidase (XO) inhibition activities of *T. diffusa* and *T. catigua* crude aqueous extracts. Results are represented as mean $\pm$ SEM from three independent assays, each performed in triplicate.

Polyunsaturated fatty acids, present at high concentrations in the human brain, easily undergo lipid peroxidation resulting in highly reactive species with a negative impact in the oxidative stress, and consequently damaging cell structures (188). Thus, *T. diffusa* and *T. catigua* aqueous extracts were also evaluated by their ability to inhibit the first stages of lipid peroxidation induced by Fe^{+2} /ascorbate system, following the formation of conjugated dienes in linoleic acid solutions (**Figure 38**). Both extracts protect linoleic acid from the induced peroxidation process, although the differences between them are not relevant ($\text{IC}_{50} = 227.18 \pm 9.04$ and 283.39 ± 34.13 μg of lyophilized aqueous extract/mL for *T. catigua* and *T. diffusa*, respectively). However, within the tested concentration range, it was only possible to fully inhibit the peroxidation of linoleic acid in the presence of *T. catigua* extract.

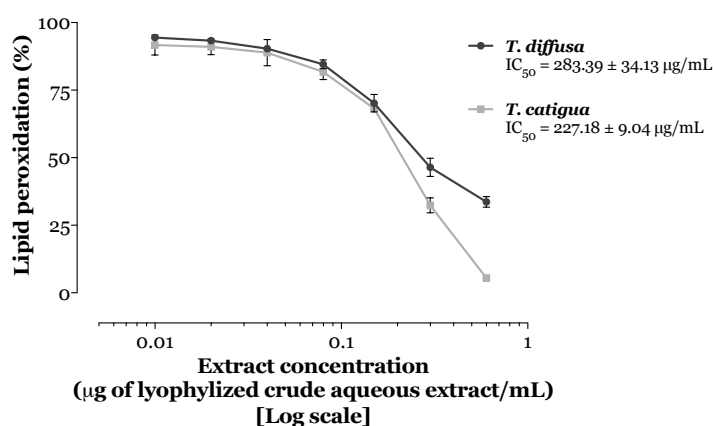


Figure 38. Inhibition of linoleic acid peroxidation by *T. diffusa* and *T. catigua* crude aqueous extracts. Results are expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

Overall, *T. catigua* antioxidant effect excels in all these assays. These results must be, at least in part, related with the phenolic content of each extract. In fact, in *T. diffusa* crude aqueous extract the quantified mass accounts for a total of 18.20 ± 2.12 mg/g of the lyophilized extract, while a much higher mass is found in *T. catigua* extract, corresponding to 551.02 ± 37.61 mg/g of the lyophilized extract.

Regarding the qualitative composition, both extracts are composed by flavonoids (**Figures 33** and **34**) featuring general structural aspects already described as key determinants to effectively display antiradical activity (**Figure 7**). Nevertheless, either for their structure, higher quantity, or combination of their effects, the compounds present in *T. catigua* display a stronger antiradical effect.

Although no single *in vitro* antioxidant activity assay will reflect the possible advantageous biological impact of antioxidants, herein is demonstrated that these extracts may contribute to neutralize the deleterious action of reactive species with relevance in the early events of neurodegenerative processes.

4.2.2. Inhibition of cholinesterases

The capacity of *T. diffusa* and *T. catigua* crude extracts to inhibit AChE and BuChE activities was also explored (**Figure 39**). In AChE inhibition assays, both extracts exhibit a concentration-dependent effect, although neither of them can completely inhibit the enzyme at the tested concentrations. The IC_{50} calculated for *T. catigua* (1.06 ± 0.01 mg/mL) is close to that of *T. diffusa* (1.33 ± 0.01 mg/mL). Similar results are observed in the BuChE assay. Once again, *T. catigua* displays a stronger inhibitory effect (**Figure 39**). These results also demonstrate that none of the extracts has a degree of selectivity for a specific cholinesterase.

Following these results, it was further assessed the kinetics of AChE inhibition in rat brain homogenates by *T. catigua* extract (**Figure 40**). In all the experimental groups, the AChE activity as a function of acetylthiocholine followed a Michaelis-Menten kinetics equation, with the values of apparent kinetic parameters (K_m and V_{max}) indicated in the insert table in **Figure 40**. All the three tested extract concentrations (50, 100 and 200 μ g/mL) inhibit AChE in rat brain homogenates, as evidenced by the increase of K_m values and by the decrease of V_{max} . The results point to a model of mix inhibition, which has the highest relevance for the typical physiological conditions that occur at the lower substrate concentrations, i.e., closer to K_m . Due to experimental limitations it was not possible to test

higher concentrations of *T. catigua* extract, since its UV absorbance interfered with the signal detection.

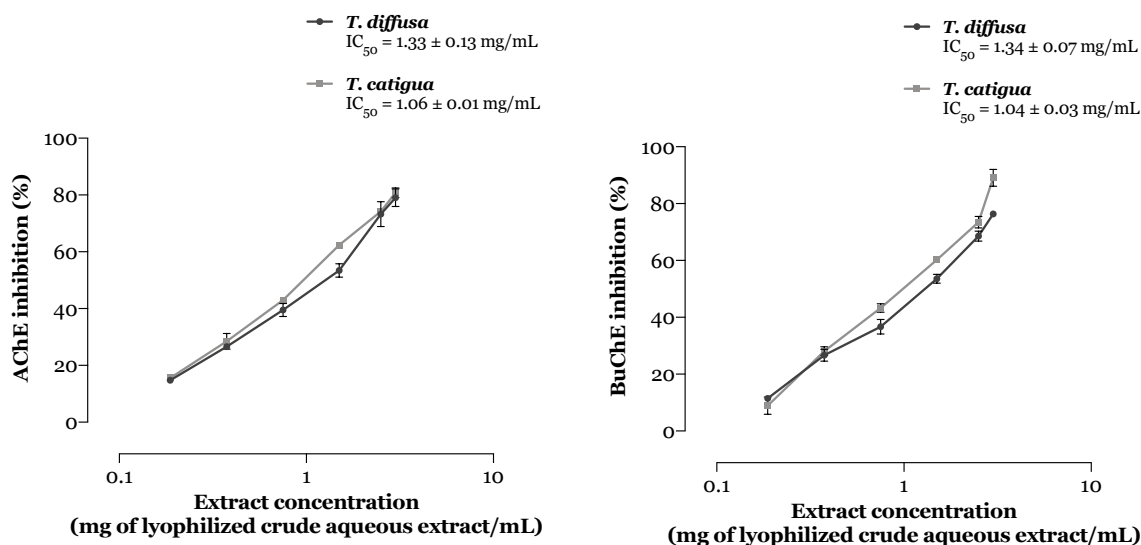
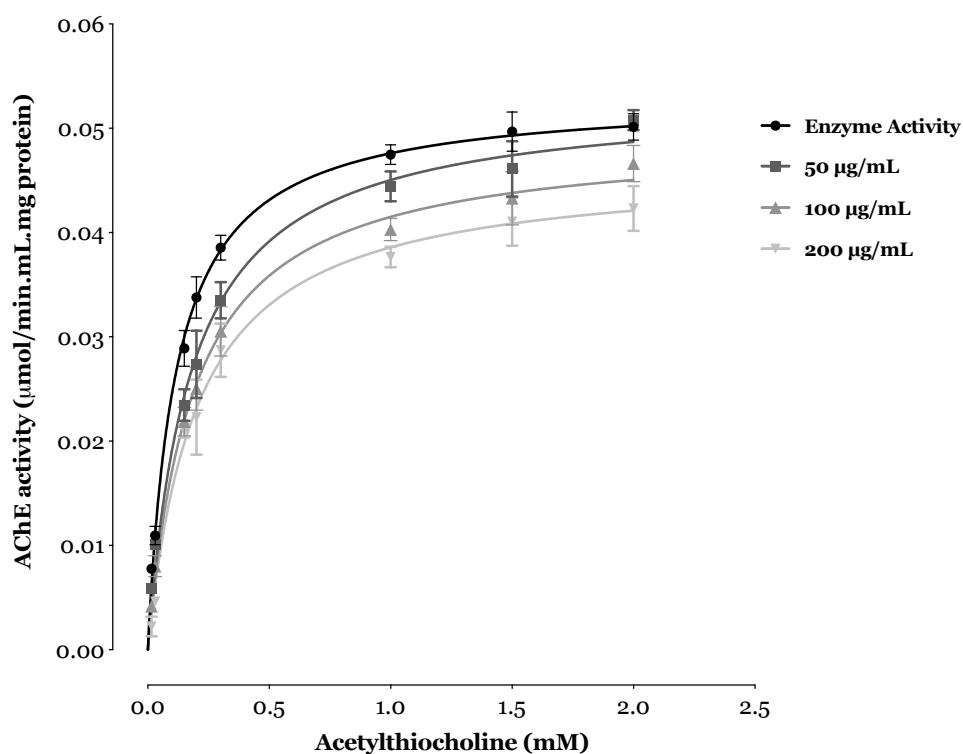


Figure 39. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activities by *T. diffusa* and *T. catigua* crude aqueous extracts. Results are expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

The available bibliography concerning the structure-activity relationships or the inhibitory effects of AChE by phenylpropanoid-substituted flavan-3-ols, the major compounds in this extract, is scarce. Nevertheless, their basic units, catechin and epicatechin, and derived compounds are known to have such effect, either isolated or when in plant extracts (395–397). Considering that *T. catigua* extract is a rich mixture of phenolic compounds, in particular flavonoids (**Figure 33**), the results obtained may derive from the effect of a single compound or from a group of the identified compounds, and the enzymatic inhibition may arise from the interaction of these compounds with multiple AChE allosteric binding sites.



	Enzymatic activity	<i>T. catigua</i> extract (μg/mL)		
		50	100	200
V_{max} (mean ± SD)	0.0531 ± 0.001	0.0529 ± 0.0016	0.0493 ± 0.0014*	0.0463 ± 0.0017*
K_m (mean ± SD)	0.116 ± 0.009	0.176 ± 0.019*	0.187 ± 0.020*	0.201 ± 0.027*
r²	0.9925	0.9858	0.9902	0.9957

Figure 40. Determination of acetylcholinesterase (AChE) activity in rat brain homogenates and its inhibition by *T. catigua* crude aqueous extract. The catalytic activity over acetylthiocholine (0.15–20.0 mM) was monitored in kinetic mode (2 min, 37 °C at 405 nm). The results are expressed as mean ± SEM from three independent assays, each performed in triplicate. Level of statistical significance *p < 0.05.

4.2.3. Inhibition of MAO-A

MAO inhibition is a way to avoid the depletion of monoamines in the synaptic cleft and simultaneously reduce the production of ROS. Therefore, the inhibition of MAO-A by *T. diffusa* and *T. catigua* crude extracts was also considered. In the tested range of concentrations, the extracts display a complete concentration-response curve (**Figure 41**). The lowest IC₅₀ was calculated for *T. catigua* (121.06 ± 2.13 μg/mL), but similar to that of *T. diffusa* extract (129.80 ± 11.97 μg/mL).

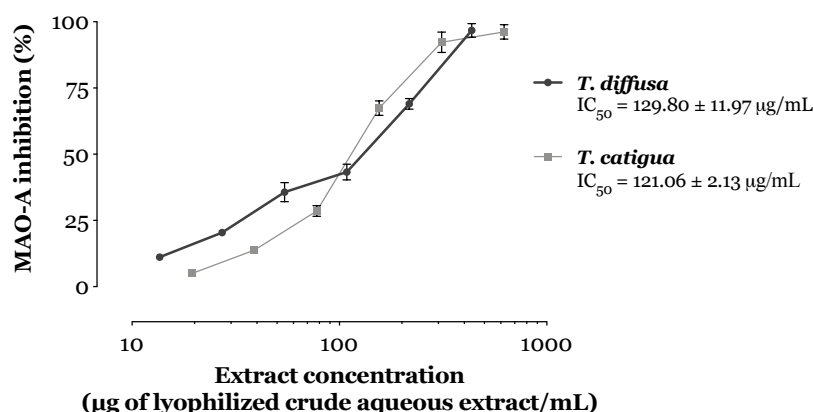


Figure 41. Inhibition of monoamine oxidase (MAO)-A by *T. diffusa* and *T. catigua* crude aqueous extracts. Results expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

In contrast with former approaches, there is a current effort to develop new therapeutic strategies comprising multiple targets that might provide greater symptomatic relief, and better results (398). For instance, there were hypothesized novel dual monoamine-cholinesterase inhibitors as potential treatment for AD (399), and simultaneous cholinesterase and brain-selective monoamine oxidase inhibitors to treat depression as comorbidity cursing with and PD (400). Furthermore, plant extracts displaying a dual enzymatic inhibitory effect may also be applied in the treatment of neurodegenerative disorders (401). Therefore, these results are relevant for both tested species.

4.2.4. Inhibition of tyrosinase

Recently, it was demonstrated that the progressive intracellular neuromelanin build-up to a certain threshold may trigger PD-like neuronal dysfunction, and neurodegeneration. It is hypothesized that, in the catecholamine-producing regions of the brain, the age-dependent accumulation of neuromelanin, a resulting oxidative downstream product from L-DOPA, courses with the overexpression of brain tyrosinase (216).

In this sense, the effects of *T. catigua* and *T. diffusa* crude aqueous extracts in tyrosinase activity were also assessed. Tyrosinase acts as monophenolase when catalyses the hydroxylation of monophenols to *O*-diphenols, and as diphenolase when oxidases *O*-diphenols to highly reactive *O*-quinones, which auto-polymerize to melanin. The tyrosinase diphenolase activity, here determined following the catalysis of L-DOPA, is significantly inhibited in the presence of the extracts in a concentration-dependent effect (**Figure 42**).

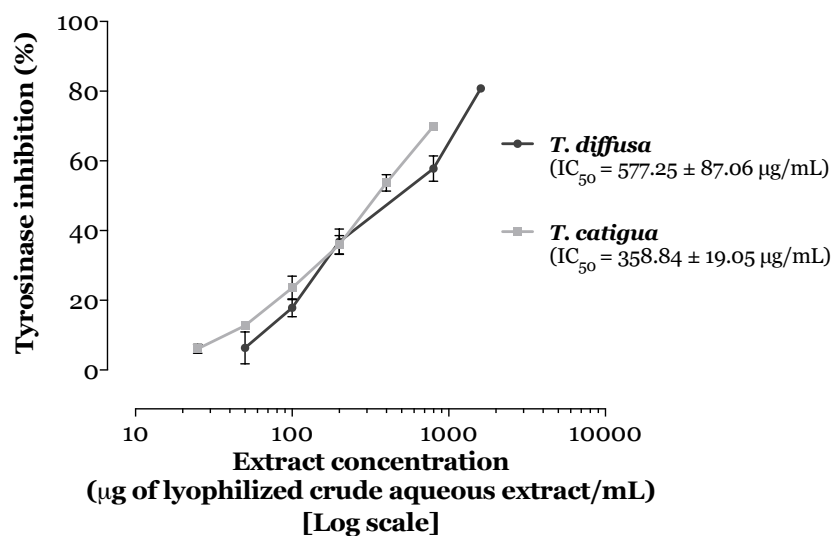


Figure 42. Inhibition of tyrosinase activity by *T. diffusa* and *T. catigua* crude aqueous extracts. Results are expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

The IC₅₀ values calculated for *T. catigua* and *T. diffusa* were of 358.84 ± 19.05 and 577.25 ± 87.06 μg of lyophilized aqueous extract/mL, respectively. Once again, the difference between the IC₅₀ values, approximately twice, may be associated to the distinct phenolic composition of each extract. *T. catigua* aqueous extract is particularly rich in flavan-3-ols (**Figure 33**), while in *T. diffusa* the dominant flavonoids are luteolin derivatives (**Figure 34**). Several flavonoids of both sub-classes are described as potent “true” tyrosinase inhibitors acting as inactivators that can form covalent bonds with the enzyme, resulting in the irreversible inactivation of the enzyme during the catalysis, or by binding to tyrosinase, thus reducing its catalytic capacity (402).

Nonetheless, due to the phenolic composition of the aqueous crude aqueous extracts, other non-direct inhibition mechanisms must be considered: the extracts may act as reducing agents, reduce back *O*-DOPAquinone to DOPA and avoid the formation of dopachrome and melanin; they may scavenge *O*-DOPAquinone and form colourless products; phenolic compounds with good affinity for the enzyme may act as substrates and the quinonoid reaction products absorb in different spectral range (403). As far as we know, this is the first work to report the inhibition of tyrosinase activity by extracts obtained from these species.

4.2.5. Antiglycation effects

As denoted before, neurodegeneration is not a linear chain of pathophysiologic events. Alongside oxidative stress and neuroinflammation, the AGEs that adversely affect the functional properties of proteins, lipids and DNA seem to have a key-role on aging, diabetes, and neurodegenerative disorders (404). The formation of AGEs is an irreversible complex multistep process. Apart from protein-AGEs, other highly reactive dicarbonyl products and ROS are formed with pro-oxidant/inflammatory activities. Moreover, transition metals can also directly oxidize the monosaccharides and form dicarbonyl products, which subsequently crosslink proteins, a process designated as auto-oxidative glycosylation.

In this sense, we explored the inhibitory effects of *T. catigua* and *T. diffusa* crude aqueous extracts on BSA-glycation (**Figure 43**). To cover for the widest types of BSA-AGEs produced by the different metabolic pathways, glycation was induced by glucose, fructose, and methylglyoxal (middle stage of glycation). The results demonstrate that both extracts can inhibit the formation of BSA-AGEs induced either by glucose, fructose and even methylglyoxal, at all concentrations (**Figure 43**). In addition, the extracts not only reduce the formation of fluorescent AGEs, as they are able to do so over 14 days (**Figure 43**).

When compared, the results obtained with the different inducers indicate that the antiglycation effects are less extent in the methylglyoxal assays (**Figure 43E and F**). After 14 days, the formation of BSA-AGEs reaches approximately 4-fold increase in the presence of *T. catigua*, and 7-fold increase in the presence of *T. diffusa* extracts, at their highest concentrations (133 µg of lyophilized aqueous extract/mL).

Considering the other two individual glycation assays, the most potent effect is displayed by *T. catigua* extract (**Figure 43A, B and C**). It is clear that, at its highest concentration it efficiently inhibits AGEs formation induced by glucose, for 14 days. Furthermore, when fructose is the glycation inducer, the same effect is observed, even at its lowest concentration (8 µg of lyophilized aqueous extract/mL). As for *T. diffusa* extract, it keeps AGEs formation to approximately 2-fold increase when glucose was the inducer, and at 6-fold increase in the fructose assay.

As far as we know, this is the first work exploring the antiglycation effects of these species. Nonetheless, flavonoids are recognized by their inhibitory effects on the formation of AGEs. In a study comprising ten flavonoids from distinct sub-classes was demonstrated that all flavonoids were active (405). Furthermore, luteolin, a flavone, was the one displaying the most relevant reduction of AGEs formation on each stage of protein glycation; among the tested flavan-3-ols, epigallocatechin gallate was the most effective

compound, and catechin and epicatechin displayed the weakest effects of all within this sequence; it was also demonstrated that the antiglycation effect was directly correlated with the free radical scavenging capacity of each flavonoid subclass, decreasing in the series flavone > flavonol > flavanol > flavanone.

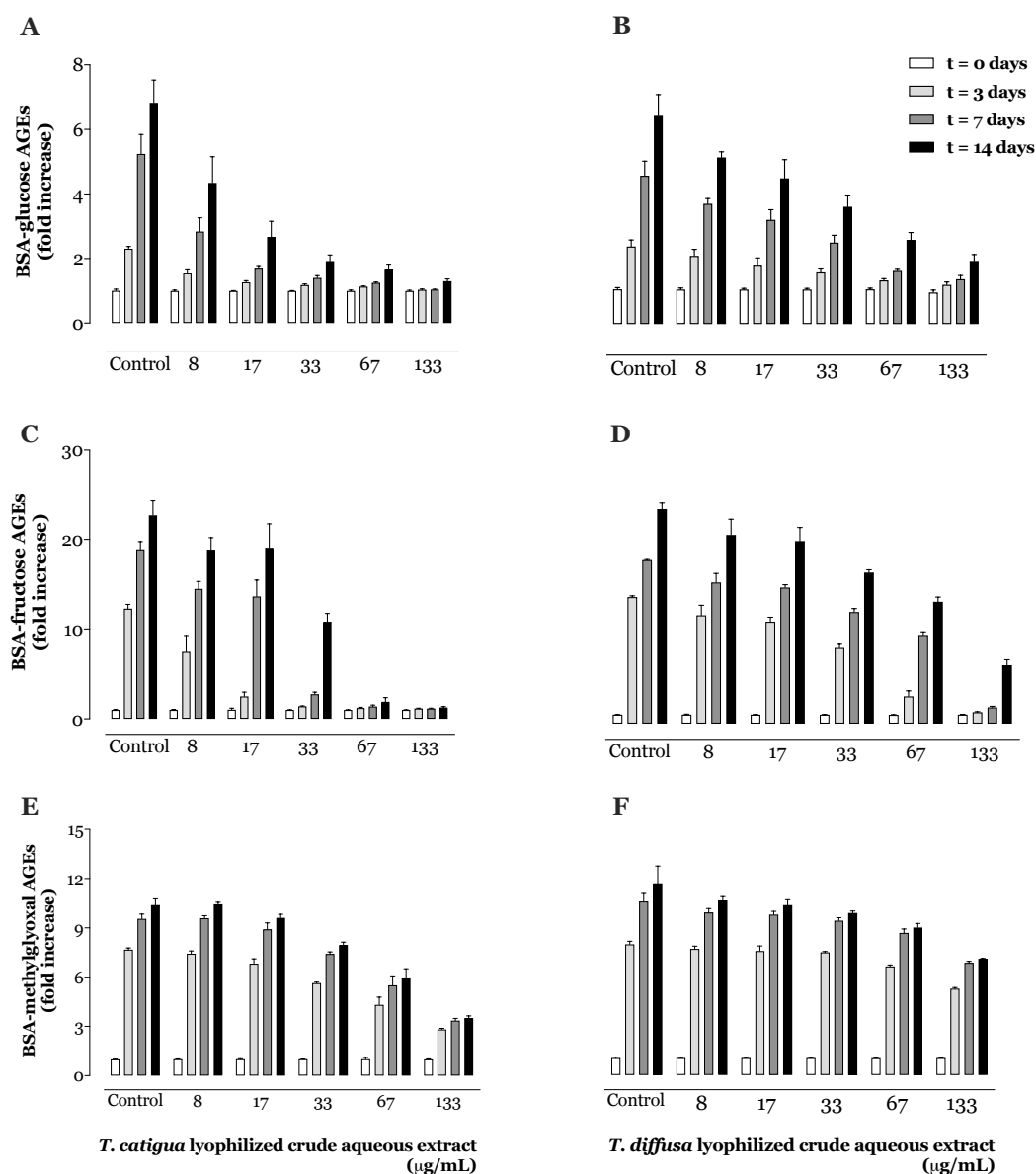


Figure 43. Inhibitory effects of *T. diffusa* and *T. catigua* crude aqueous extracts on BSA-glycation induced by glucose (A and B), fructose (C and D) and methylglyoxal (E and F), for 14 days. Results are expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

Nonetheless, in this work *T. catigua* extract excels when compared to *T. diffusa*, but it must be stressed that most flavonoids in the extracts are present in their glycosylated forms, rather than as aglycones, and possible synergic effects cannot be disregarded.

Moreover, since diabetes relates to chronic neurodegenerative disorders, this mechanism may help to provide a protective effect against hyperglycaemia-mediated protein damage (406).

4.2.6. Protective effects against neuronal-induced cytotoxicity

The neuroprotection exerted by flavonoids comprehends more than the direct antiradical activity, modulation of the monoaminergic and cholinergic systems and inhibition of enzymes. As discussed before, they are also able to trigger endogenous antioxidant mechanisms, interact with signalling pathways, or even to protect neurons from glutamate toxicity. The exacerbation of glutamate stimulus can trigger a phenomenon described as “excitotoxicity” that is associated with chronic neurodegenerative disorders. Increasing evidence shows that glutamate and mitochondria are two prominent players in the establishment of cell damaging oxidative stress (407).

In this sense, the capacity of an extract to protect neurons against glutamate toxicity may, thereby, constitute a valuable feature and promote its application as neuroprotective agent. To further explore the neuroprotective potential of *T. diffusa* and *T. catigua* extracts, their effects against glutamate-induced toxicity was studied in the neuronal model SH-SY5Y. The exact mechanism driving glutamate toxic effects over SH-SY5Y cells is still not clear, some authors suggesting an effect mediated by glutamate receptors, while others support that these cells are deficient in these receptors; also accepted, is the huge burst in ROS production that results in cell death (408).

First, it was assessed the effect of the extracts over cell viability by the MTT reduction test to ensure that they do not cause cytotoxic effects. In the tested range of concentrations (1–200 µg of extract/mL) none of the two extracts affected the viability of the neuroblastoma SH-SY5Y cells (**Figure 44A and B**).

In contrast, and as expected, the cell treatment with glutamate decreased cell viability to ~75%. Pre-treatment of cells with *T. catigua* and *T. diffusa* extracts fails to protect them against glutamate-induced toxicity (**Figure 44C and D**). However, the pre-treatment of these cells with either extract protected them from 6-OHDA/AA induced toxicity (**Figure 44E and F**). The protective effect of *T. catigua* extract is more preponderant because it was observed at a lower concentration.

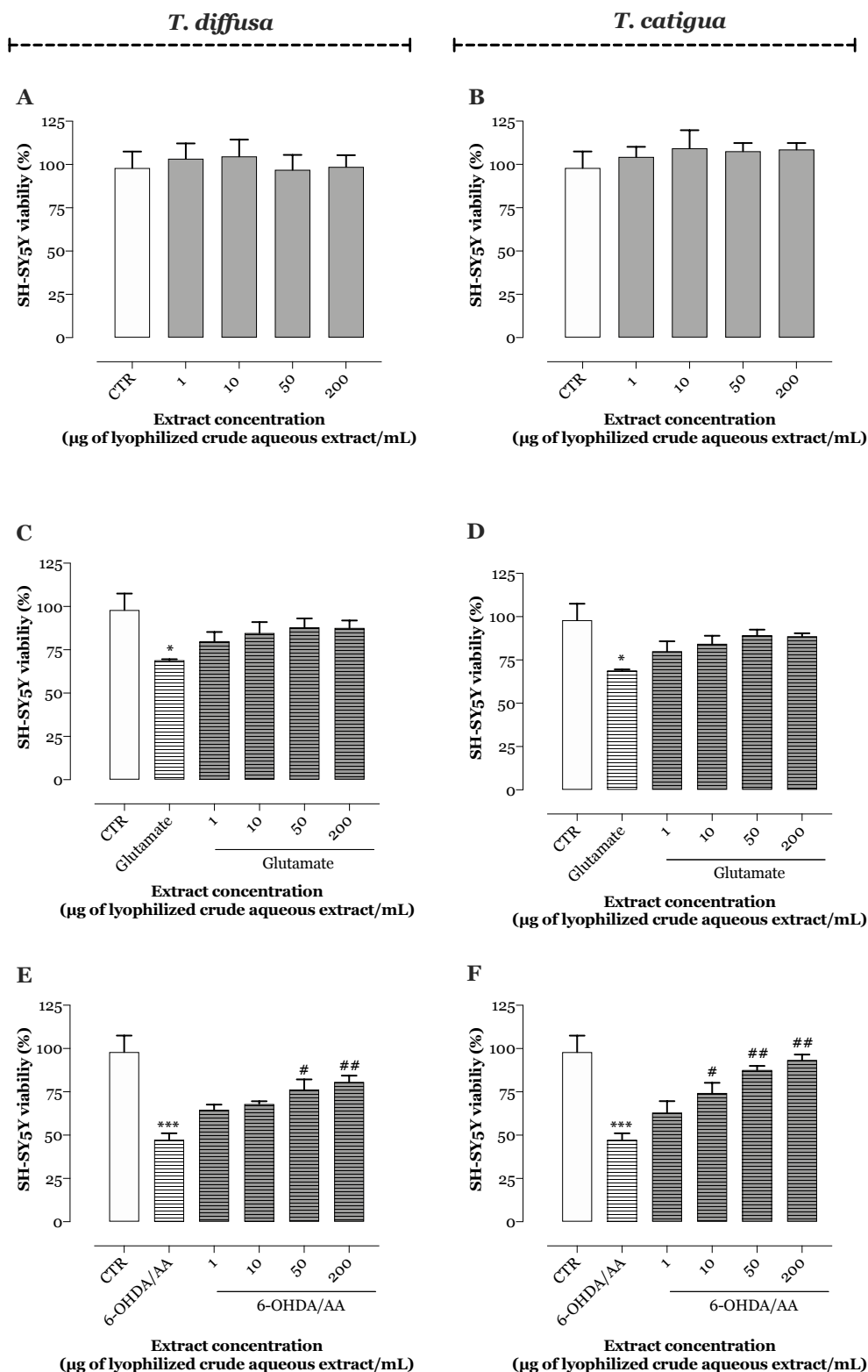


Figure 44. Performance of *T. catigua* and *T. diffusa* crude aqueous extracts in SH-SY5Y neuroblastoma cell model. In the tested range of concentrations, both extracts do not alter cell viability (A and B). The pre-treatment of cells with the extracts fails to protect them from glutamate (50 mM) (C and D). In contrast, the pre-treatment with the extracts protects the cells from 6-hydroxydopamine/ascorbic acid (6-OHDA/AA; 100 mM + 0.02 %) (E and F). The results are expressed as mean $\pm$ SEM from five independent assays, each performed in triplicate. The statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* (level of statistical significance * $p < 0.05$). * and ***, significantly different from the control with $p < 0.05$ and $p < 0.001$, respectively; # and ##, significantly different from cells exposed to the toxicants with $p < 0.05$ and $p < 0.01$.

4.2.7. Effects on enzymes and pathways engaged in the neuroinflammatory process

Neuroinflammation is also deeply connected to neurodegenerative disorders since a chronic low-grade inflammatory process interferes with the homeostasis of all the CNS elements and has a causal role in neurodegeneration (410). Therefore, the modulation of effectors or targets engaged in this process may be useful to prevent or delay harmful effects. Lipoxygenases are crucial elements within the inflammatory response. They catalyse the oxidation of free and esterified polyunsaturated fatty acids, such as arachidonic and docosahexaenoic acids, forming bioactive lipids responsible for the progression of inflammation, and with significant impact in the course of neurodegenerative diseases. Despite that 12/15-LOX are the most abundant isoforms in the CNS, age-dependent upregulation of the 5-LOX pathway is extremely relevant in cortex and hippocampus, two areas known to be particularly vulnerable to neurodegeneration (263).

The effects of *T. catigua* and *T. diffusa* aqueous extracts on 5-LOX activity were also assessed. A concentration-dependent effect for both extracts is observed, and 5-LOX activity is completely inhibited at the maximum tested extract concentrations (**Figure 45**). The calculated IC_{50} for *T. diffusa* extract is of 136.23 ± 25.38 μ g of lyophilized aqueous extract/mL. *T. catigua* extract is approximately twice effective ($IC_{50} = 56.25 \pm 14.53$ μ g of lyophilized aqueous extract/mL).

In the available bibliography we could not find any other reference reporting 5-LOX inhibition by any type of extract obtained from these species. However, it is reported that an hydroalcoholic extract of *T. catigua* decreased human platelets phospholipase A2 (PLA2) activity in a concentration-dependent effect, which is considered to be pivotal enzyme in inflammation (411). Therefore, *T. catigua* extract may display anti-inflammatory effects not only by blocking LOX pathway, but also by inhibition of PLA2 activity upstream in the inflammatory process, with the consequent blockage of LOX and cyclooxygenase pathways.

Together with the direct inhibition of enzymes engaged in the inflammatory process, the extracts are also capable of scavenging nitric oxide radical (**Figure 46**). The calculated IC_{50} values are 231.66 ± 10.97 and 285.45 ± 11.81 μ g of lyophilized aqueous extract/mL for *T. catigua* and *T. diffusa* extracts, respectively. As previously discussed, the suppression of reactive species, either oxygen or nitric derivative ones, may have positive impact in the cell redox status. In addition, and because $\cdot NO$ is also an inflammation mediator, these results point to a possible anti-inflammatory potential of the extracts.

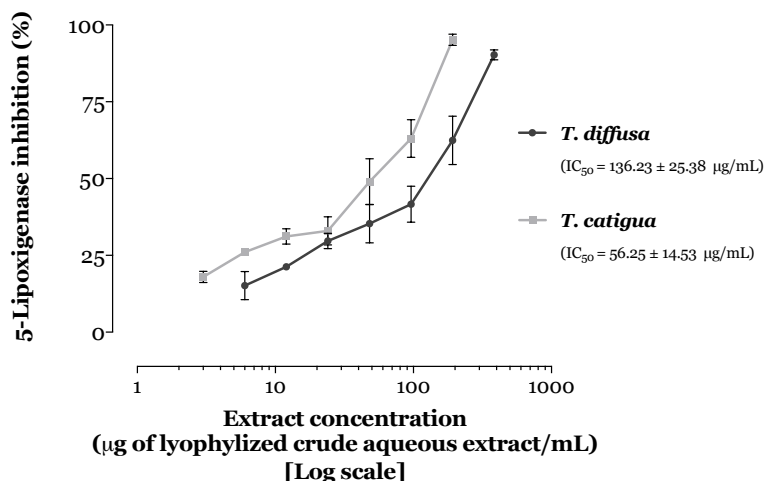


Figure 45. Inhibition of 5-lipoxygenase activity by *T. diffusa* and *T. catigua* crude aqueous extracts. Results are expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

The inflammatory response in the CNS is essentially mediated by activated microglia through different, and yet complementary, complex mechanisms. The chronic activation of microglia results in the production of pro-inflammatory cytokines, proteases, and NO and ROS. Therefore, the suppression or mitigation of the microglia-mediated inflammation is considered as an important strategy in neurodegenerative disease therapy (412).

There is no currently available *in vitro* method that capitulates all hallmarks of homeostatic microglia, neither mimic human neuroinflammatory response. Nevertheless, the murine microglial cell line BV-2 has shown to exhibit some appropriate morphological, phenotypical, and functional properties of microglial cells, including independent induction of iNOS by IFN- γ (413).

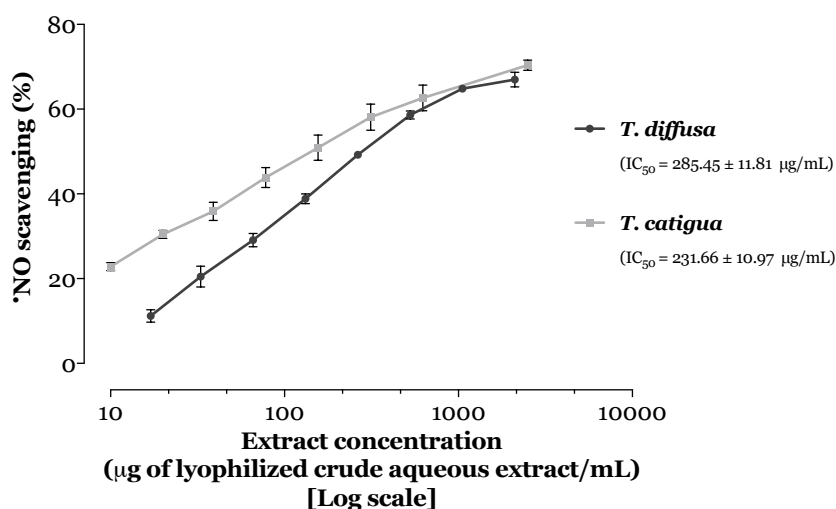


Figure 46. Scavenging of nitric oxide radical by *T. diffusa* and *T. catigua* crude aqueous extracts. Results are expressed as mean $\pm$ SEM from three independent assays, each performed in triplicate.

The effects of *T. catigua* and *T. diffusa* extracts on BV-2 viability were studied by using the MTT reduction assay (**Figure 47A and B**). It is demonstrated that, within the range of concentrations tested, none of the extracts affects BV-2 cell viability, neither in the co-treatment with IFN- γ .

NO release is frequently assessed to follow the neuroinflammatory response mediated by microglia, BV-2 cells included, since it is an important inflammatory mediator with implications in tissue injury and cell death in many neurodegenerative diseases (262). In BV-2 cells, IFN- γ insult simulates an immune response triggered by cytokines, which leads to an increased NO production as result of the induction of iNOS expression *via* ERK1/2 cascade (414).

The impact of the extracts on BV-2 cells NO production was evaluated, in terms of nitrite levels, in the cell culture supernatants obtained from non-stimulated and IFN- γ -stimulated cells (**Figure 47C and D**). The extracts were tested at the same concentration range, and *per se* none was able to increase NO production compared to that of cells in basal conditions (control) (**Figure 47C and D**). In contrast, and as expected, cell stimulation with IFN- γ triggered a significant increase of the cellular NO production to values 50% higher than basal levels (**Figure 47C and D**).

The two extracts led to different effects. *T. catigua* extract failed to mitigate the IFN- γ induced inflammatory stimulus (**Figure 47C**) despite its remarkable antioxidant activity, including NO radical scavenging. On the other hand, *T. diffusa* extract displayed a concentration-dependent effect (**Figure 47D**), since at 50 and 200 μ g of lyophilized aqueous extract/mL it was able to reduce cellular NO production by 35 and 40%, respectively, when BV-2 cells were stimulated with IFN- γ .

Also relevant, *T. diffusa* extract has a lowest phenolic content than that of *T. catigua*. However, it is the one that protects BV-2 cells from IFN- γ . This effect can emerge from the putative effects on iNOS expression, direct effects on iNOS activity, as well as from the modulation of complex processes underlying microglia activation by IFN- γ stimulus, which seems to be independent of the NF- κ B pathway. The modulation of NO pathways by *T. diffusa* aqueous extract is already supported by one previous work reporting its pro-sexual effect in rats involving the participation of the NO pathway, mainly at central level (362).

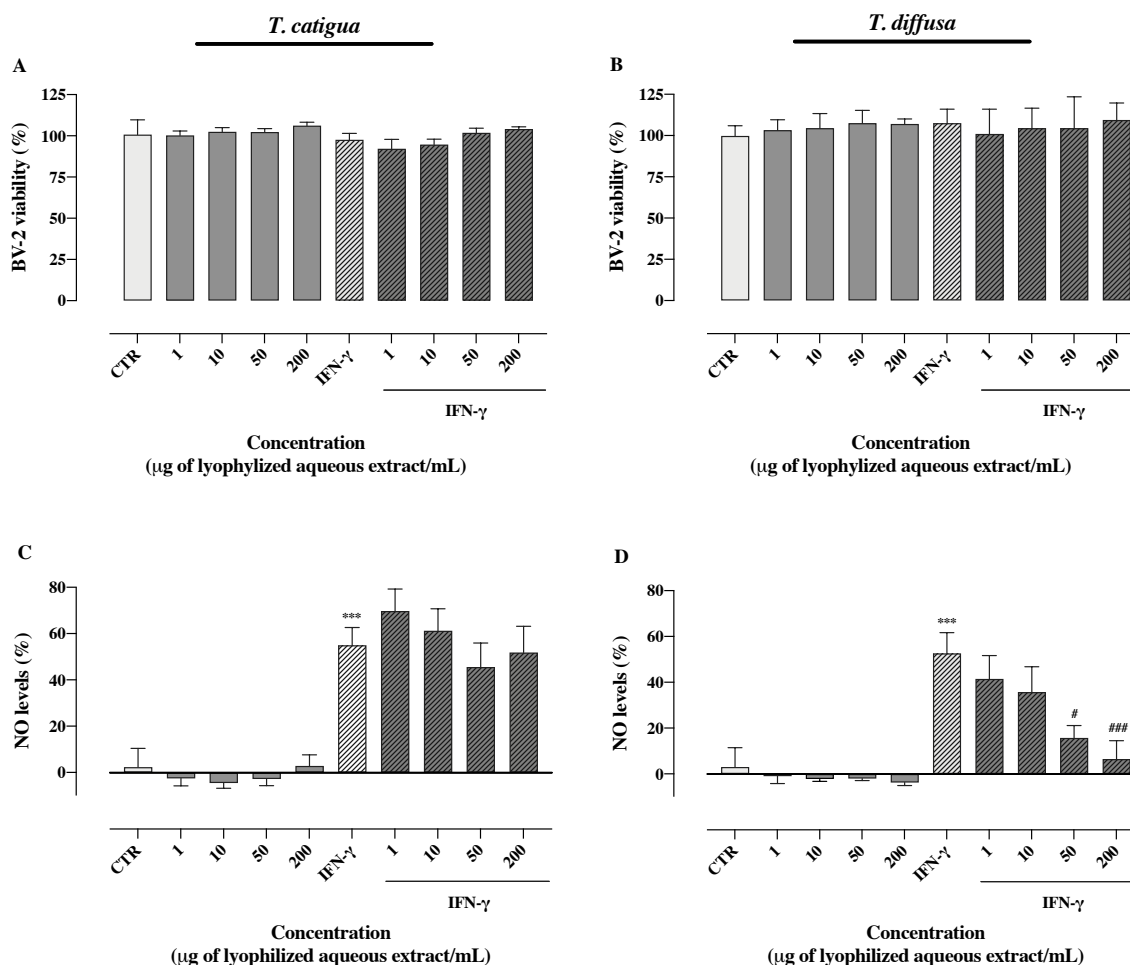


Figure 47. Performance of *T. catigua* and *T. diffusa* crude aqueous extracts in BV-2 microglia cells. Viability of BV-2 cells assessed by MTT reduction assay (A and B), and quantification of NO levels in culture supernatants by Griess reaction (C and D). Results obtained from untreated cells, from cells treated with the extracts or insulted with IFN- γ (20 U/mL), and from cells pre-treated with the extracts followed by insult with IFN- γ . The results were expressed as mean $\pm$ SEM from five independent assays, each performed in triplicate. The statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* (level of statistical significance * $p < 0.05$). ***, significantly different from the control with $p < 0.001$; # and ###, significantly different from the cells exposed to IFN- γ with $p < 0.05$ and $p < 0.001$, respectively.

The microglial priming by IFN- γ represents one of the mechanisms leading to excessive ROS production with subsequent neuronal damage (415). In particular, NO overproduction by activated glia triggers calcium mobilization from the endoplasmic reticulum of astroglia cells and results in glutamate-mediated neurotoxicity. This is one additional reason why targeting NO production by glial activation has been suggested as a helpful strategy to halt aging and neurodegenerative diseases. In this way, *T. diffusa* effects on BV-2 cells are valuable within a new therapeutic approach for neurodegeneration aiming to target microglial activation.

According to all the results presented in this section, it can be concluded that, as expected, the distinct phenolic composition of *T. catigua* and *T. diffusa* crude aqueous extracts has impact over their biological activities. Regarding the attempt to correlate the bioactivities with the chemical nature of the extracts, it is hard to get objective conclusions because these are complex and integrative biological processes that involve numerous biochemical reactions, targets, and effectors. It would not be reasonable a compound-effect discussion, as these extracts are mixtures, and their final effect results from synergic/antagonist interactions that are not possible to single out. Furthermore, even if *T. catigua* extract has a higher phenolic content, it does not have to correspond to a stronger biological effect. Despite that *T. catigua* extract excelled in all the *in vitro* cell-free bioactivities, only *T. diffusa* was able to attenuate NO production induced by IFN- γ activation of BV-2 microglia cells. Nonetheless, their antioxidant and antiglycation effects, inhibition enzymes, neuronal protection against induced cytotoxicity and interaction with pathways engaged in neuroinflammation may add a valuable input to how these species may help to counteract neurodegenerative processes. In addition, all these results highlight the value of plant extracts, especially the ones rich in flavonoids, and provides preliminary data to support and validate the traditional medicinal use of these two species.

4.3. Nanoliposomes and nanophytosomes: production, characterization, and study of their neuroprotective effects

As discussed before, flavonoid-based therapies at an appropriate stage of neurodegeneration may hold promise to halt neurodegenerative processes and to improve cognitive function, with positive health outcomes. Nonetheless, the low brain bioavailability of polyphenols was considered as one of the major pitfalls behind their application. On this basis, the production of liposomes entrapping flavonoids or flavonoid-rich extracts may enhance their bioavailability, allowing them reaching cell targets in pharmacological effective concentrations.

4.3.1. Extraction of phospholipids from egg yolk

Egg yolk is a rather interesting natural source of bioactive compounds, of easy access and at low expenses. In addition, with the recent advances in the areas of animal feeding and nutrition, eggs have a high potential as functional food due to the possibility to produce designer eggs on demand (416). However, the partition of lipids from egg yolk, like the

extraction of phospholipids, is significantly hampered by the varied composition of natural supramolecular assemblies of lipids and proteins in this matrix (417). One of the recurrent issues of extracting phospholipids from egg yolk is the traditional utilization of chloroform (418), an organic solvent known by its toxicity at low concentrations (419). Consequently, its presence in phospholipid extracts, even in residual amounts, hinders their application in conceivable formulations with potential therapeutic and food applications. On the other hand, the extraction protocols themselves are often not adequately versatile, nor viable for the scale-up required by industry.

Taking in consideration all this knowledge, here is proposed a new protocol to obtain standardized phospholipid-rich fractions from egg yolk. When a phospholipid-directed extraction is intended, it becomes necessary, right at the outset, to separate lipids from yolk proteins, including from those with high-affinity for lipids (i.e., disassembling lipoproteins), and then separate phospholipids from the other lipids. To overcome this issue, it was outlined a multi-step procedure to obtain egg yolk phospholipids in rich fraction using ethanol and acetone, two solvents approved for food applications (**Figure 20**).

Ethanol is used uppermost to disassemble lipoproteins and to promote protein delipidation and precipitation, thus allowing efficient extractions with high yields (419). With this first step, the total lipids are collected in the ethanol fraction. At the same time, the solid yolk residue, dominated by aggregates of denaturised proteins, can be collected by centrifugation, and be used for re-extraction, or simply discarded. The ethanol fraction is then concentrated by evaporation under reduced pressure to obtain a solution of extractable lipids at 1 g egg yolk/0.5 mL ethanol (**Figure 20**).

This concentrated complex mixture of egg yolk lipid-base compounds, containing 240 ± 10.50 μmol of phospholipids/g fresh egg yolk, is further submitted to crystallization/precipitation for 14 h, at 4 °C. The temperature drop triggers the crystallization and/or precipitation of the lipids with lower solubility in cold ethanol (e.g., neutral lipids), such as triacylglycerols (420), which can be collected by centrifugation. On the other hand, polar lipids, including phospholipids, cholesterol, and carotenoids, remain soluble in the supernatant (ethanol), here designated as polar lipid-rich fraction (**Figure 20**). In this step, it was taken advantage from the distinct solubility of different groups of lipids in cold ethanol to discard the neutral ones. Notably, in the next step, it is the distinct solubility of polar non-ionic lipids and ionic lipids in cold acetone that enables to obtain phospholipids (ionic lipids) in a separate fraction (421).

With the evaporation of ethanol, followed by wash-up cycles with cold acetone, is obtained a lipid precipitate, collected by centrifugation, which is mainly constituted by

phospholipids and herein designated as phospholipid-rich fraction (**Figure 20**). The cold acetone solubilizes the triacylglycerol surplus, high amounts of cholesterol and other non-ionic lipids, such as carotenoids (canthaxanthin, β -carotene, lutein, and zeaxanthin). By these means, it is obtained a cholesterol and carotenoid-rich fraction after acetone evaporation (**Figure 20**). In contrast, the resulting phospholipid-rich fraction has a significantly lower amount of cholesterol, and traceable lutein and zeaxanthin, more polar oxycarotenoids, as revealed by the chemical characterization described below.

4.3.2. Chemical characterization of the phospholipid-rich fraction

Four phospholipid-rich fractions, obtained from independent egg yolks, were characterized considering their phospholipid profile (total content and fatty acid composition), cholesterol content and carotenoid profile (identification and quantification of molecular species), to evaluate the efficiency of the process and the degree of purity of the final extract.

4.3.2.1. Phospholipids and fatty acids

Data in **Table 10** indicate that the extraction procedure is efficient, being translated into high yields along with a high degree of reproducibility, as demonstrated by the narrow variation of the phospholipid content of the four independent samples analysed ($208.65 \pm 26.46 \mu\text{mol/g}$ fresh egg yolk).

Phospholipids account for approximately 96% of the fraction dry weight. In addition, when expressed as percentage of fresh egg yolk weight, the mean of extraction yields is around 16%. These findings fit the goals, since similar results were reported when applying a multi-step protocol for an exhaustive large-scale extraction of phospholipids from egg yolk using ethanol and hexane (422).

Table 10. Characterization of the four-individual phospholipid-rich fractions (normalized to fresh egg yolk weight), each obtained from independent extractions.

	Extraction number				Mean $\pm$ SD ^c
	1	2	3	4	
Fresh egg yolk weight (g)	37.566	38.710	36.388	39.596	38.065 $\pm$ 1.393
Phospholipids (μ mol/g fresh egg yolk) ^a	222.80 $\pm$ 6.21 (18%) ^b	184.33 $\pm$ 10.04 (15%) ^b	188.67 $\pm$ 3.06 (15%) ^b	238.81 $\pm$ 8.11 (17%) ^b	208.65 $\pm$ 24.74 (16%)
Cholesterol (μ mol/g fresh egg yolk) ^a	16.95 $\pm$ 3.71	13.38 $\pm$ 0.55	13.08 $\pm$ 0.55	19.57 $\pm$ 1.12	15.75 $\pm$ 3.27
Lutein (ng/g fresh egg yolk) ^a	15.52 $\pm$ 1.29	16.34 $\pm$ 4.31	16.14 $\pm$ 0.38	11.73 $\pm$ 4.34	13.81 $\pm$ 2.48
Zeaxanthin (ng/g fresh egg yolk) ^a	91.07 $\pm$ 9.40	98.46 $\pm$ 1.67	91.98 $\pm$ 6.33	75.46 $\pm$ 5.13	88.33 $\pm$ 11.75
Cholesterol/ phospholipid molar ratio	0.076	0.073	0.069	0.082	0.075 $\pm$ 0.005
Molar fraction of cholesterol (%)	7.07	6.77	6.48	7.57	6.97 $\pm$ 0.47

^a Expressed as mean $\pm$ SD, calculated from determinations of three aliquots of each independent extraction.

^b % of extraction = (phospholipid (g)/fresh egg yolk (g) $\times$ 100).

^c Calculated considering all the single determinations.

However, the utilization of hexane as extractant is a disadvantage since putative residual amounts of this solvent confer toxic risk to the final products. A more efficient egg yolk phospholipid extraction yield (~30%) was reported by using a protocol with ethanol to extract total lipids, a precipitation step at low temperatures to discard neutral lipids, and β -cyclodextrin to remove cholesterol (423). Nonetheless, the success of this methodology is dependent on a step that requires temperatures close to 50 °C and the use of β -cyclodextrin. Therefore, this step entails higher expenses than our proposed procedure; likewise, it significantly increases the risk of the polyunsaturated fatty acids present in egg yolk phospholipids to undergo oxidation, which has a negative impact in food and pharmacological applications.

Apart from validating the efficiency of the process, it is also important to characterize the PL profile of the final fraction. Egg yolk phospholipids have been extensively explored, being mainly constituted by PC and PE, accounting up to 76 and 22% of total phospholipids,

respectively (417). Trace amounts of lysoPC and lysoPE, phosphatidylinositol, phosphatidylserine, sphingomyelin, or cardiolipins can also be found in egg yolk lipid extracts. In this work, the qualitative analysis of each final phospholipid-rich fraction was performed by TLC. In the chromatogram are clearly visible two major spots with retardation factors similar to those of EggPC and DPPE. This indicates that PC and PE are also the main classes in the fraction.

The fatty acid composition of the extracted phospholipids was also evaluated since it has biological relevance for further uses of these fractions in food and pharmaceutical industries. It is well-documented that the fatty acid composition of the egg yolk phospholipids can be modulated by the diet fed to hens, which has impact on the relative abundance of PUFA and monounsaturated fatty acids (MUFA), as well as in the *n*-6/*n*-3 PUFA molar *ratio* (424). In general, saturated fatty acids (SFA) account for 45-50% of egg yolk PL, while the MUFA levels range between 20 to 25%, and PUFA can reach up to 35% (417).

Figure 48 and **Table 11** show the fatty acid profile of the phospholipids found in the final fractions obtained by the proposed procedure. Thirteen fatty acids are identified and quantified, comprising four SFA, two MUFA and seven PUFA. Despite the wide diversity of PUFA molecular species, SFA palmitic acid (C_{16:0}) is always the one with the highest relative abundance (31.91 ± 0.79%), followed by stearic acid (C_{18:0}; relative abundance of 18.25 ± 2.22%). Consequently, the fatty acid profile of the extracted egg yolk PL is dominated by saturated fatty acids, representing ~51% of the total content. As for the other fatty acid classes, total MUFA represent around 29% of the fatty acid content of the extracted PL, oleic acid (C_{18:1}) being much more abundant than palmitoleic acid (C_{16:1}) (**Table 11**).

On the other hand, PUFA represent 21% of the phospholipid fatty acid content; among those, linoleic acid (C_{18:2} *n*-6) is the most representative of *n*-6 PUFA series, and eicosapentaenoic acid (C_{20:5} *n*-3) dominates within the *n*-3 PUFA. The *n*-6/*n*-3 PUFA *ratio* calculated for the phospholipid-rich fraction is of 1.8±0.1 (**Table 11**).

This fatty acid profile complies with previously reported data (425), and is an indication that the fraction is dominated by membrane phospholipids. In general, PC and PE classes exhibit a saturated fatty acid in *sn*-1 position and a monounsaturated or a polyunsaturated fatty acid in the *sn*-2 position of the glycerol, promoting profiles with 50% of saturated fatty acids. It was already reported that in the fatty acid profile of egg yolk PC, approximately 33% are C₁₆, 14-20% are saturated C₁₈, and around 40% are unsaturated C₁₈ (mainly oleic acid) (425).

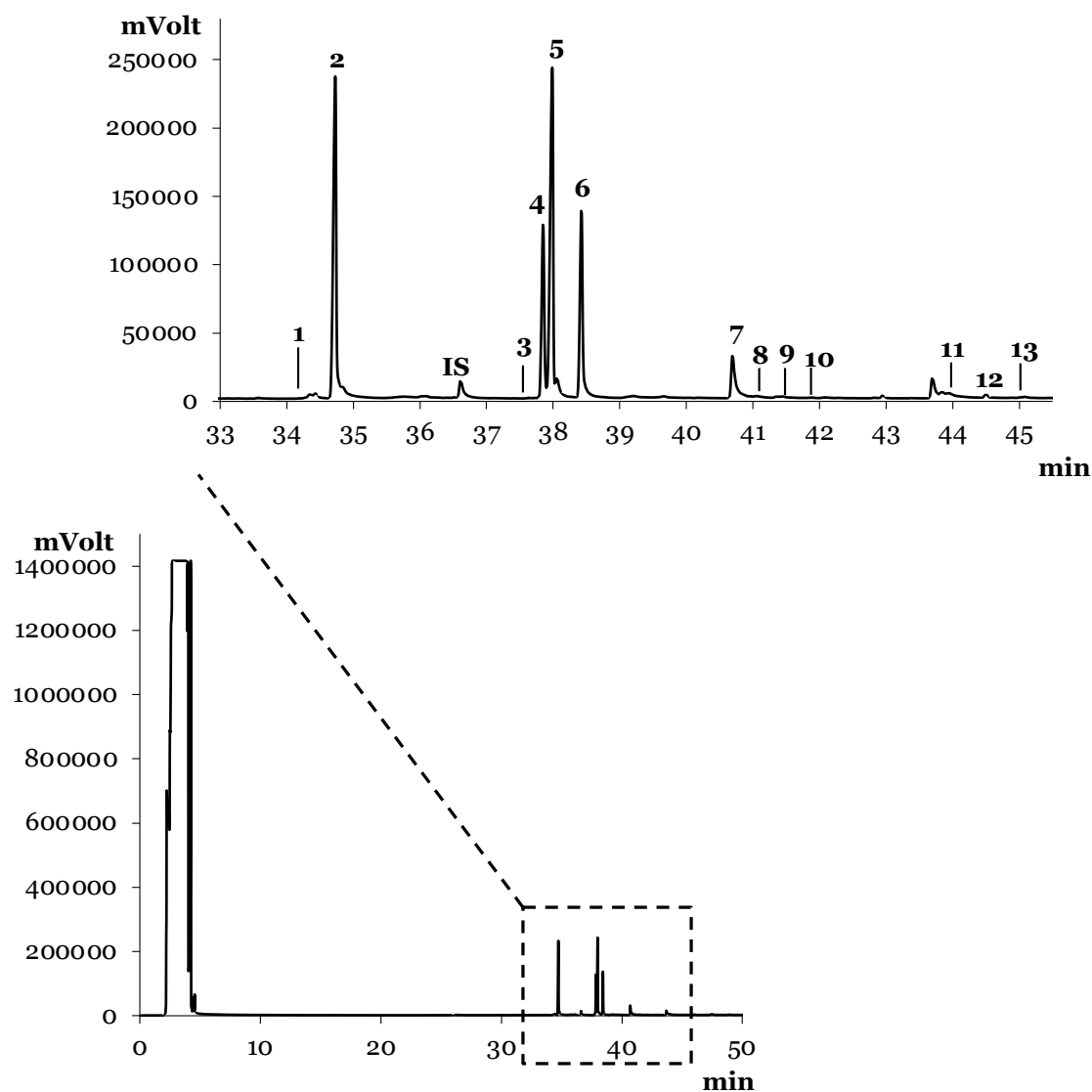


Figure 48. Representative GC-FID chromatogram of the fatty acid composition of phospholipids extracted from egg yolk, after acid hydrolysis. **1:** C_{16:1}; **2:** C_{16:0}; **3:** C_{18:3 n-3}; **4:** C_{18:2 n-6}; **5:** C_{18:1}; **6:** C_{18:0}; **7:** C_{20:5 n-3}; **8:** C_{20:3 n-3}; **9:** C_{20:2 n-6}; **10:** C_{20:0}; **11:** C_{22:6 n-3}; **12:** C_{22:2}; **13:** C_{22:0}; **IS:** Internal standard, C_{17:0}.

These results also sustain that triglycerides are removed during the extraction procedure (**Figure 20**), since their fatty acid distribution contents is of approximately 60% of oleic (C_{18:1}) and linoleic (C_{18:2}) acids (426).

Table 11. Fatty acid composition of the phospholipid-rich fractions

Fatty acid ^a	Phospholipid-rich fraction				Mean $\pm$ SD ^b
	1	2	3	4	
C _{16:1}	1.04	0.69	0.84	0.94	0.88 $\pm$ 0.15
C _{16:0}	31.70	31.04	32.94	31.94	31.91 $\pm$ 0.79
C _{18:3 n-3}	0.10	0.06	0.10	0.05	0.08 $\pm$ 0.03
C _{18:2 n-6}	12.90	12.83	11.84	12.59	12.54 $\pm$ 0.49
C _{18:1}	25.32	30.99	25.86	29.60	27.94 $\pm$ 2.78
C _{18:0}	21.39	16.77	18.26	16.58	18.25 $\pm$ 2.22
C _{20:5 n-3}	5.68	5.45	5.60	5.15	5.47 $\pm$ 0.23
C _{20:3 n-3}	0.26	0.53	0.59	0.78	0.54 $\pm$ 0.21
C _{20:2 n-6}	0.01	0.13	0.17	0.19	0.13 $\pm$ 0.08
C _{20:0}	0.18	0.11	0.31	0.14	0.19 $\pm$ 0.09
C _{22:6 n-3}	0.91	0.83	0.87	0.99	0.90 $\pm$ 0.07
C _{22:2}	0.09	0.37	1.55	1.37	0.85 $\pm$ 0.72
C _{22:0}	0.43	0.19	0.98	0.53	0.53 $\pm$ 0.33
Σ SFA (%)	~54	~48	~52	~48	51 $\pm$ 3
Σ MUFA (%)	~26	~32	~27	~31	29 $\pm$ 3
Σ PUFA (%)	~20	~20	~21	~21	20 $\pm$ 1
<i>n-6/n-3 PUFA ratio</i>	1.9	1.9	1.7	1.8	1.8 $\pm$ 0.1

^a Mean of determinations of three independent derivatizations obtained from each phospholipid-rich fraction, expressed as relative % of the internal standard C_{17:0}.

^b Calculated considering all single determinations.

4.3.2.2. Cholesterol content

In general, protocols implementing the sequential partition of egg yolk lipids are delineated to obtain phospholipid extracts of maximum purity by discarding the unwanted by-products, such as cholesterol. Nonetheless, cholesterol itself plays a strategic role in the functional organization of biological membranes and in the phospholipid-based formulations. In fact, the lateral organization of phospholipids (e.g., membranes rafts dynamics), fatty acyl chain fluidity, membrane permeability and the partition of lipophilic drugs are dependent upon the relative levels of cholesterol (427). Thus, cholesterol levels higher than 10% are commonly used in lipid-based formulations to increase the drug encapsulation efficiency, to improve the stability of the vesicles, or even to obtain a most favourable drug release pattern within the biological systems (428).

The total cholesterol content in the phospholipid-rich fraction is displayed in **Table 10**. The relative abundance of cholesterol, expressed as molar percentage, ranged between 6.48 and 7.57%, and the cholesterol/phospholipids molar *ratio* varied between 0.069 and 0.082. Thus, the extraction protocol exhibits good reproducibility, and the levels of cholesterol are relatively low, which suggests that the fractions can be used, without further purification steps, to prepare lipid-based formulations for food and pharmacological applications. However, it is also important to stress that the cholesterol content in the final fraction can be adjusted, if, and when necessary, by increasing or reducing the number of precipitation/wash-up steps with cold acetone (**Figure 20**), representing an additional advantage of the proposed methodology.

4.3.2.3. Lutein and zeaxanthin content

The chemical characterization of the phospholipid-rich fractions, obtained from egg yolks by the proposed protocol (**Figure 20**), would not be complete without considering its content of carotenoids. In fact, egg yolk carotenoids, likewise cholesterol, are considered by-products and undesirable impurities of intended phospholipid-pure extracts; notwithstanding, their presence in lipid nanostructures may constitute an asset.

The HPLC-DAD analysis of the PL-rich fraction revealed trace amounts of two oxycarotenoids, lutein and zeaxanthin (**Figure 49A**). Similar levels of these two xanthophylls were found in all the final fractions (**Table 10**), obtained from four independent egg yolk samples, supporting again the reproducibility of this extraction protocol. Zeaxanthin is the one present at higher concentration, followed by lutein, with mean $\pm$ SD values of 89.24 ± 9.76 and 14.93 ± 2.16 ng/g of fresh egg yolk, respectively. Lutein is systematically described as the dominant carotenoid in egg yolk, being found in a 2:1 *ratio* relative to zeaxanthin (429); the same *ratio* was found in the final lipid extracts when the egg yolk was fractionated with ethanol and hexane, but the ethanolic extraction of zeaxanthin was demonstrated to be more efficient than the one of lutein (430). Therefore, the results here obtained can be explained, at the same time, by the low temperature effect and the distinct solubility of xanthophylls in ethanol/acetone.

Carotenoids, and their enzymatic cleavage products, are associated with diverse biological processes with a positive impact on human health, and are widely implemented in industry, as food ingredients, additives, or supplements (431).

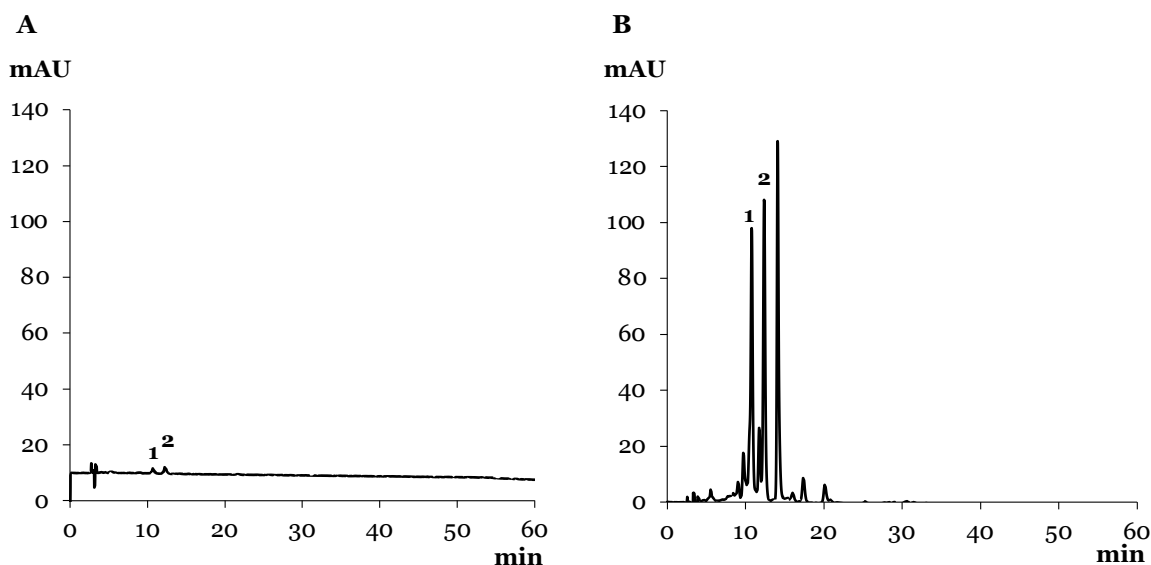


Figure 49. Representative HPLC-DAD chromatograms (at 450 nm) of the carotenoids in the phospholipid-rich fractions (A), and in the collected acetone fractions (B).

Their broad versatility also versers their application in nanotechnology, either by encapsulation in liposomes to enhance their biological activities (432), or by the impact of the carotenoid–lipid interactions on the dynamics of the lipid bilayers (433). As extensively reviewed, polar carotenoids, such as lutein and zeaxanthin, are proved modifiers of the structural and dynamic properties of phospholipid membranes, which ultimately will increase the quality of liposome products, by spanning lipid bilayers with their polar groups anchored in opposite zones of the membrane; they favour the stiffness of the fluid phase of the phospholipid membranes due to the van der Waals interactions with the acyl chains of lipids; they limit oxygen penetration and act as antioxidants, hence preventing chemical degradation reactions by the hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and the peroxidation of unsaturated acyl chains (434).

As for the other characteristic carotenoids of egg yolk, such as canthaxanthin, β -carotene, capsanthin, β -cryptoxanthin, among others (429), they are not detected in these final fractions (**Figure 49A**). For most carotenoids, non-ionic lipids, it is expected a more efficient qualitative and quantitative extraction with a relatively less polar solvent (acetone), rather than with a polar one (ethanol). In fact, this is confirmed by the HPLC-DAD analysis of the acetone fractions, which shows the presence of numerous peaks corresponding to carotenoids, lutein and zeaxanthin being found among them (**Figure 49B**). Apart from the main goal, and since acetone fractions can be collected and preserved, these by-products can be latter separated and redirected towards multiple applications.

Overall, the outlined experimental procedure allows extracting phospholipids from egg yolk using only ethanol and acetone, following the European guidelines for extraction solvents used in the production of food and pharmaceutical formulations. With this procedure are obtained phospholipid-rich fractions of high purity degree without compromising the extraction yields. It was also designed to minimize the consumption of solvents by recovering ethanol for continuum extraction cycles. Therefore, this procedure aggregates valuable features for a possible industrial scale-up. Moreover, the main by-products, cholesterol, and carotenoids, are obtained in separated acetone solutions, thus allowing their further application. Finally, the PL-rich fraction can be applied to produce lipid-based formulations.

4.3.3. Application of the phospholipid-rich fraction as building-block material for rutin-nanoliposomes

The standardized egg yolk phospholipid-rich fraction here obtained can be used as main ingredient in several types of commercial lipid-based products, including in the smart nano-systems used to deliver pharmacological drugs and nutraceuticals. One possibility is to produce liposomes to entrap flavonoids or flavonoid-rich extracts, thus increasing their CNS bioavailability and enhancing their neuroprotective effects.

In this work, rutin (quercetin-3-*O*-rutinoside) is selected as flavonoid-model compound to be entrapped in liposomes once it is widely found in the diet (435), and exhibits a bioactivity with potential to promote positive impacts on degenerative brain diseases (436).

To that purpose, the phospholipid-rich fraction used to produce rutin-nanoliposomes is mainly composed by phospholipids at the concentration of 945.59 $\mu\text{mol/mL}$, being dominated by saturated fatty acids distributed by ~24% of monounsaturated and ~35% of polyunsaturated. The cholesterol molar fraction is ~8% and residual amounts of lutein (46.45 ng/mL) and zeaxanthin (298.79 ng/mL) are present.

4.3.3.1. EE, size distribution and Zeta potential

The amount of rutin entrapped in liposomes increases linearly with increasing bulk flavonoid concentrations in the buffer solution used to prepare the liposomes (**Table 12**). However, the EE decreases with decreasing phospholipid:rutin *ratios* and reaches its maximum of $87.0 \pm 0.9\%$ at 80:1, which is the highest tested *ratio*. In fact, data of EE

suggest that the encapsulation of rutin in liposomal formulations follows a saturation curve (**Table 12**), as reported for other flavonoids (437).

Besides the EE, the mean particle size and Zeta potential of the nanoformulations are also affected by the amount of rutin entrapped within the lipid membranes. The blank liposomes present a mean particle size of 101.0 ± 8.2 nm, while liposomes entrapping rutin, at all loading concentrations, display higher values (**Table 12**). This phenomenon was already described for other liposomes loading flavonoids, in particular, for those loading the parent aglycone, quercetin (437). In terms of Zeta potential, as the rutin entrapped concentration increases, the surface charge is progressively more negative (**Table 12**). Since strong electronegative values are normally predictive of stable nanoformulations (438), these results indicate that rutin entrapment improves the physical stability of liposomes.

Table 12. Entrapment efficiency (EE), mean particle size, polydispersity index (PDI), and Zeta potential of blank nanoliposomes and nanoliposomes entrapping rutin

	EE (%) ^a	Mean particle size (nm) ^a	PDI ^a	Zeta potential (mV) ^a
Blank nanoliposomes		101.0 ± 8.2	0.259 ± 0.080	-5.72 ± 0.70
Liposomes + Rutin 250 μ M	66.8 ± 2.0 (167 μ M) ^b	138.2 ± 30.4	0.286 ± 0.006	-13.19 ± 2.45
Liposomes + Rutin 125 μ M	68.6 ± 2.1 (86 μ M) ^b	133.2 ± 12.1	0.305 ± 0.007	-12.98 ± 2.65
Liposomes + Rutin 63 μ M	80.0 ± 1.4 (50 μ M) ^b	138.0 ± 12.6	0.293 ± 0.007	-11.39 ± 2.93
Liposomes + Rutin 32 μ M	87.0 ± 0.9 (28 μ M) ^b	128.3 ± 10.0	0.290 ± 0.005	-10.09 ± 2.83

^a Results represent the mean $\pm$ SD from three independent determinations.

^b Final concentration of rutin entrapped in liposomes predicted by the EE.

4.3.3.2. Interaction of rutin with liposome membranes

The characterization of rutin-lipid membrane interactions is an essential topic because some of the physicochemical parameters of the nanoformulation, including size, formulation stability, surface charge, and, consequently, its biological activity will be conditioned by them. Thus, the partition coefficient of rutin between water and lipid membranes, as well as its preferential localization across the bilayer thickness, were investigated by fluorescence quenching methods, using the 16-AP membrane probe. In liposomes, the 16-AP molecule has a preferential vertical orientation, and its anthroyloxy moiety is located deeply in the hydrocarbon region of the phospholipid bilayer (439). Therefore, if a molecule directly changes the fluorescence characteristics of this environment-sensitive fluorophore (i.e., maximum wavelength, fluorescence intensity or lifetime), then it penetrated the phospholipid bilayer, thus being located close to the hydrophobic core.

The fluorescence intensity of 16-AP decreases with increasing concentrations of rutin up to 50 μM (**Figure 50**), which suggests that the flavonoid is deeply incorporated in the lipid bilayer. Thus, rutin interacts with the hydrophobic chains of phospholipids, despite its 3-*O*-rutinose polar substitution. Moreover, the fluorescence quenching data can be described by Stern-Volmer equation, and the reciprocal of the apparent Stern-Volmer constant ($1/K_{app}$) increases linearly with the increasing volume of lipid membranes (from 100 to 600 μM in phospholipid), allowing the determination of the partition coefficients (K_p) for rutin (table insert in **Figure 50**). The highest K_p value obtained for rutin ($K_p = 1230 \pm 187$) indicates that the flavonoid is preferentially accumulated into the hydrophobic core of the lipid membrane. Therefore, it can promote changes in the lipid packing order and membrane fluidity with positive impact on the stability of liposomes and surface charge, as previously suggested for other flavonoids (440).

Taking this data in consideration, apparently the nanoliposomes produced with the egg yolk phospholipid-rich fraction are a valuable platform to entrap other flavonoids, besides rutin, or even flavonoid-rich extracts obtained from medicinal species.

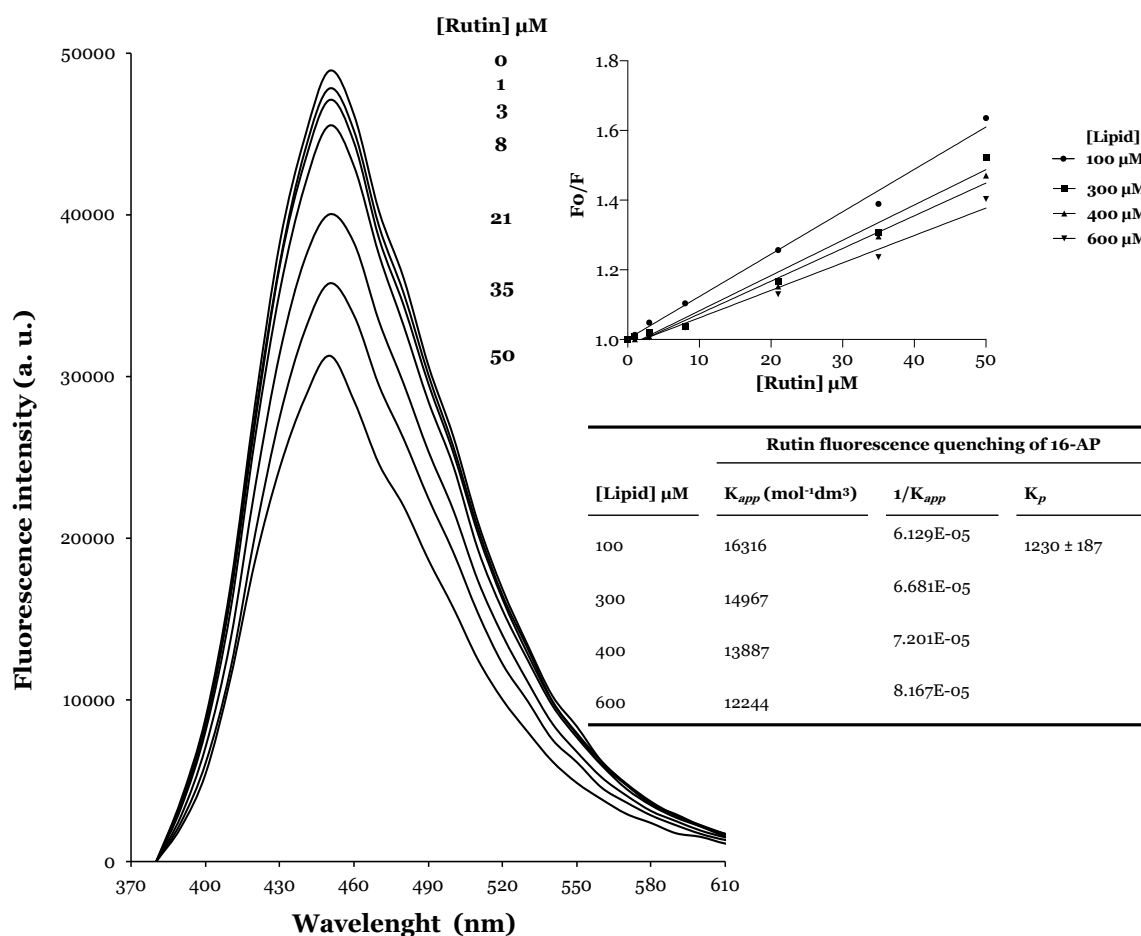


Figure 50. Fluorescence emission spectra ($\lambda_{exc} = 365\text{nm}$) of 16-AP probe incorporated in liposomes (100 μM of lipid from the phospholipid-rich fraction) in the absence and presence of increasing concentrations of rutin (1–50 μM). In the inserted graphic are the Stern-Volmer plots from steady-state fluorescence measurements with 16-AP incorporated in increasing lipid concentrations (100–600 μM) as function of rutin concentration (0, 1, 3, 8, 16, 21, 35 and 50 μM). In the inserted table are the calculated values of the Apparent Stern-Volmer Constant (K_{app}) and of the partition coefficient (K_p) of rutin incorporated in liposomes, at 37 °C, at different lipid concentrations.

4.3.3.3. Rutin-nanoliposomes protect SH-SY5Y cells against glutamate induced toxicity

As discussed before, mitochondrial dysfunction and elevated ROS production are downstream toxicological endpoints of glutamate-induced toxicity, and these are all interconnected key-elements underlying neurodegenerative processes.

Therefore, it was also explored the neuroprotective effect of rutin-nanoliposomes against glutamate-induced toxicity in SH-SY5Y cells. In the first step, the effect of rutin-nanoliposomes over the cell viability was determined by the MTT reduction test. The results demonstrate that the cell pre-treatment with rutin-nanoliposomes (phospholipids 25 μM + rutin 16.7 μM) do not reduced cell viability (**Figure 51**). On the other hand, when compared

to the untreated control, the cell exposition during 22 h to glutamate (50 mM) is responsible for reducing cell viability to ~65% (**Figure 51**). In addition, when cells were exposed to glutamate for 1 h, is observed an increased production of intracellular reactive species up to more ~45% of the levels of the control (**Figure 51**). These results support that mitochondrial dysfunction and elevated ROS production are deleterious effects caused by glutamate-induced toxicity in SH-SY5Y cells.

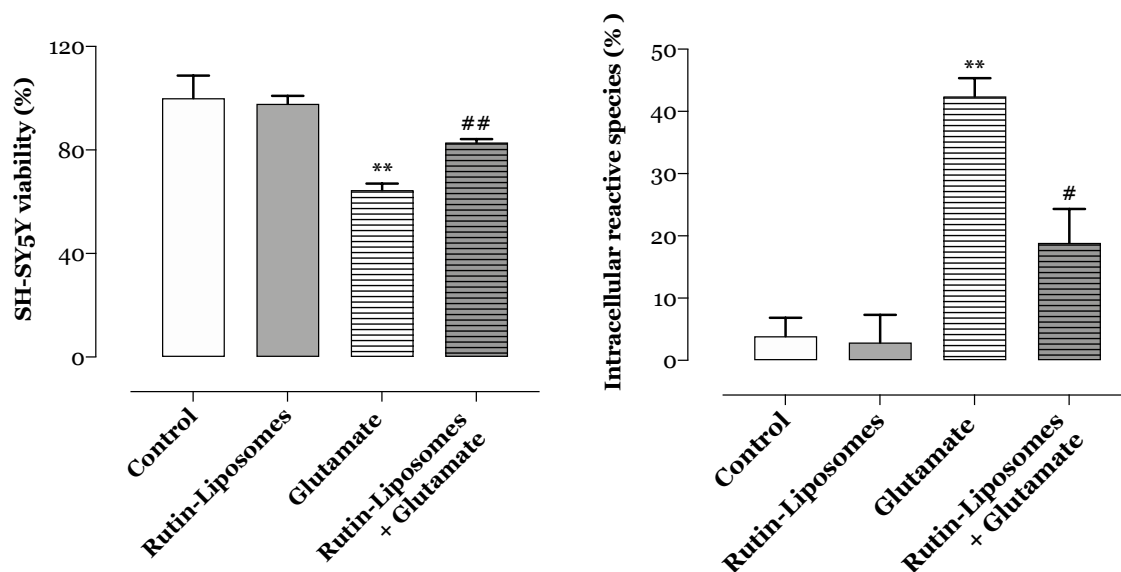


Figure 51. Performance of rutin-nanoliposomes in the neuronal SH-SY5Y cell line. The cell viability was assessed by MTT reduction after treatment with rutin-nanoliposomes (phospholipids 25 μ M + rutin 16.7 μ M), glutamate (50 mM), and after pre-treatment with rutin-nanoliposomes followed by co-exposition with the toxicant. Intracellular reactive species were determined following the same treatment conditions. The results were expressed as mean $\pm$ SEM from five independent assays, each performed in triplicate. The statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* (level of statistical significance * $p < 0.05$). **, significantly different from control with $p < 0.01$; # and ##, significantly different from the cells exposed to glutamate with $p < 0.05$ and $p < 0.01$, respectively.

The pre-treatment of SH-SY5Y cells with rutin-nanoliposomes (phospholipids 25 μ M + rutin 16.7 μ M) for 2 h protects them from the exposition to glutamate, as observed by the cell viability levels that under these experimental conditions are maintained at ~80% (**Figure 51**). This protective effect may be, at least in part, mediated by antioxidant mechanisms, since the pre-treatment with rutin-nanoliposomes significantly reduces to ~20% the levels of intracellular reactive species triggered by glutamate. These results agree with a previous work reporting that rutin was able to attenuate glutamate-induced ROS production in several neuronal cell lines, and to protect SH-SY5Y cells from glutamate-induced excitotoxicity (441).

Taking in consideration the nano size, negative surface charge, and neuroprotective effect against glutamate-induced toxicity of rutin-nanoliposomes, this nanoformulation may come as an interesting strategy to be further explored within the field of neuroprotective agents. Furthermore, the good results in terms of flavonoid entrapment suggest that the phospholipid-rich fractions may be good lipid carriers for other flavonoids or flavonoid-rich extracts.

4.3.4. *T. diffusa* and *T. catigua* nanophytosomes

To produce *T. catigua* and *T. diffusa* nanophytosomes was used one of the phospholipid-rich fractions obtained from egg yolk in combination with flavonoid-rich extracts obtained from each species by SPE. As before, the fraction is mainly composed by phospholipids at the concentration of 945.59 $\mu\text{mol/mL}$, being dominated by saturated fatty acids distributed by ~24% of monounsaturated and ~35% of polyunsaturated. The cholesterol molar fraction is ~8% and residual amounts of lutein (46.45 ng/mL) and zeaxanthin (298.79 ng/mL) are present.

The characterization of the nanoparticles, in terms of chemical composition, shape, size, surface charge and aggregation, is provided in the following sections.

4.3.4.1. Phenolic profile of *T. diffusa* and *T. catigua* flavonoid-rich extracts

The SPE procedure was implemented to remove, as much as possible, non-flavonoid compounds from *T. catigua* and *T. diffusa* lyophilized crude aqueous extracts.

The respective yields were calculated, and from the initial 200.00 mg of each extract are recovered 160.11 ± 2.11 mg (~80%) from *T. catigua* and 91.90 ± 3.21 mg (~46%) from *T. diffusa*.

The HPLC-DAD analysis allowed to identify the same twenty-six compounds in *T. catigua* flavonoid-rich extract (**Figure 52A**) that were previously identified in the initial crude extract (**Figure 33**). Likewise, thirty-seven compounds identified in *T. diffusa* flavonoid-rich extract (**Figure 52B**) correspond to those identified in the initial crude extract (**Figure 34**) Therefore, by using SPE the surplus of non-phenolic compounds is washed away, keeping the integrity of the phenolic qualitative composition.

The total quantified mass in *T. catigua* flavonoid-rich extract is of 621.97 ± 36.41 $\mu\text{g}/\text{mg}$ of flavonoid-rich extract (**Table 13**) with nineteen flavonoids (**4**, **8–24** and **26**) representing 99%. In *T. diffusa* flavonoid-rich extract is quantified a total mass of 205.73 ± 32.02 $\mu\text{g}/\text{mg}$ of flavonoid-rich extract, with the fifteen luteolin derivatives (**29** and **41–54**) accounting for ~49%, and ~38% corresponding to the eleven apigenin derivatives (**27**, **31**, **33**, **39**, **47** and **55–60**).

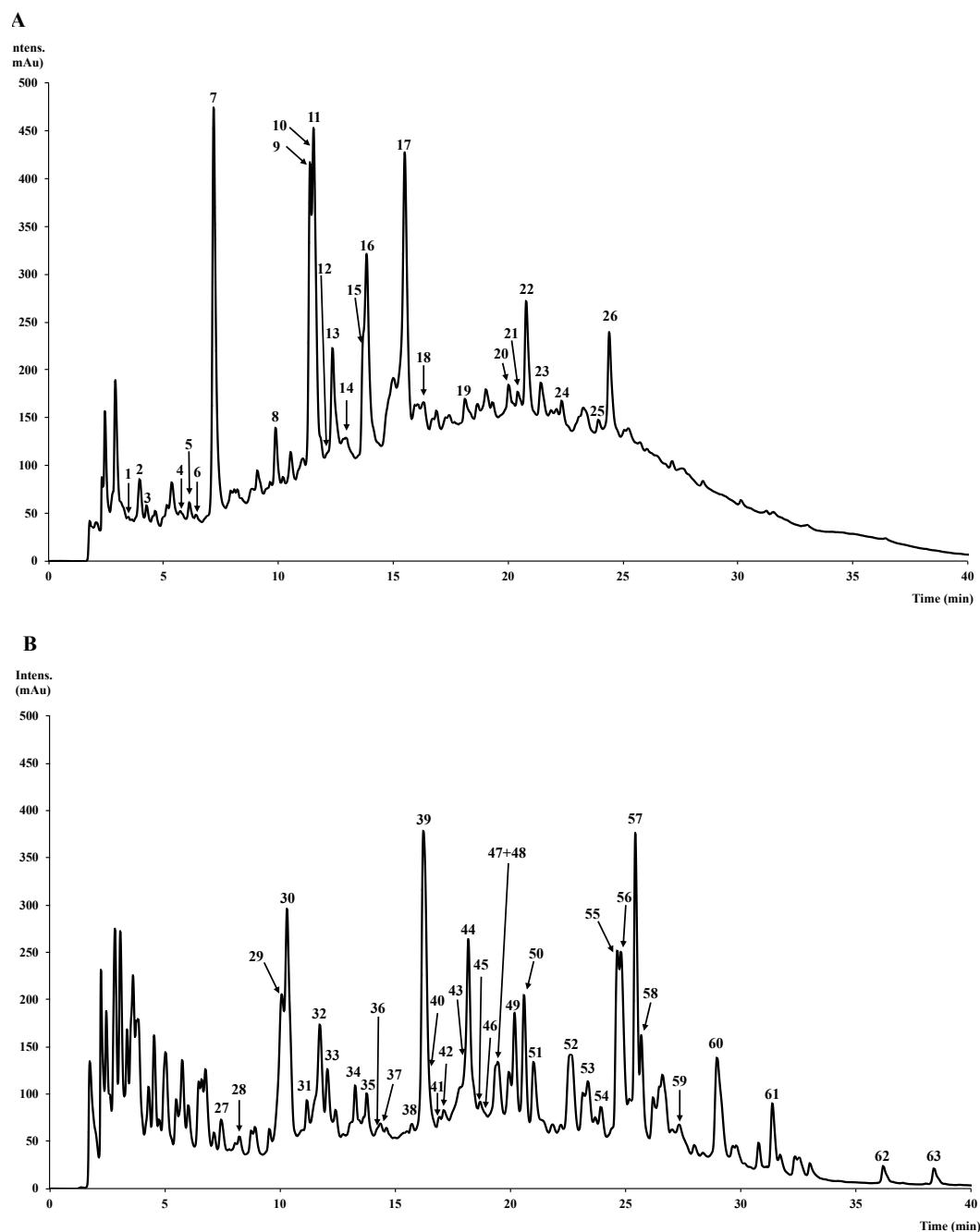


Figure 52. Chromatograms HPLC-DAD chromatograms of the flavonoid-rich extracts of *T. catigua* (at 280 nm) (A) and of *T. diffusa* (at 350 nm) (B). Identity of compounds as in **Figure 33** and **Figure 34**, respectively.

Table 13. Quantification of phenolic compounds identified in *T. catigua* and *T. diffusa* flavonoid-rich extracts.

<i>T. catigua</i>		<i>T. diffusa</i>	
Compound ^a	µg/mg of phenolic-rich extract ^b	Compound ^a	µg/mg of phenolic-rich extract ^b
1	1.12 ± 0.13	27	4.78 ± 0.38
2	0.69 ± 0.13	28	3.45 ± 0.53
3	1.20 ± 0.21	29	2.03 ± 0.03
4	5.85 ± 1.04	30	2.08 ± 0.07
5	0.71 ± 0.02	31	0.68 ± 0.16
6	0.74 ± 0.09	32	0.45 ± 0.05
7	1.50 ± 0.13	33	1.23 ± 11.61
8	1.17 ± 0.02	34	11.61 ± 0.39
9	27.14 ± 4.74	35	3.28 ± 0.07
10	2.25 ± 0.29	36	1.54 ± 1.16
11	27.52 ± 2.14	37	3.18 ± 0.68
12	28.92 ± 7.02	38	2.70 ± 0.37
13	5.52 ± 0.94	39 + 40	14.81 ± 3.38
14	7.68 ± 1.18	41	3.86 ± 0.19
15	113.29 ± 5.95	42	7.65 ± 1.03
16	129.05 ± 3.24	43	1.56 ± 0.03
17	7.99 ± 0.61	44	42.32 ± 7.15
18	8.44 ± 0.19	45	3.59 ± 0.06
19	6.01 ± 1.70	46	0.65 ± 0.01
20	5.71 ± 2.43	47 + 48	8.15 ± 0.29
21	6.49 ± 2.34	49	8.58 ± 1.74
22	5.48 ± 0.28	50	1.40 ± 0.86
23	8.02 ± 0.44	51	6.85 ± 2.71
24	9.21 ± 1.04	52	3.69 ± 0.10
25	0.58 ± 0.12	53	0.48 ± 0.11
26	209.72 ± 18.55	54	1.86 ± 0.64
Σ^c	621.97 ± 36.41	55	12.11 ± 2.31
		56	3.81 ± 0.46
		57	15.21 ± 1.08
		58	4.70 ± 1.48
		59	8.80 ± 1.63
		60	13.72 ± 0.19
		61	2.37 ± 0.37
		62	2.12 ± 1.80
		63	1.44 ± 0.25
		Σ^c	205.73 ± 32.02

^a Identity of compounds as in **Figure 33** and **Figure 34**.^b Results expressed as mean ± SD from three independent determinations.^c Sum of the quantified compounds.

These *T. catigua* and *T. diffusa* flavonoid-rich extracts were the ones combined with the phospholipid-rich fraction extracted from egg yolk to produce the nanophytosomes (**Figure 53**).

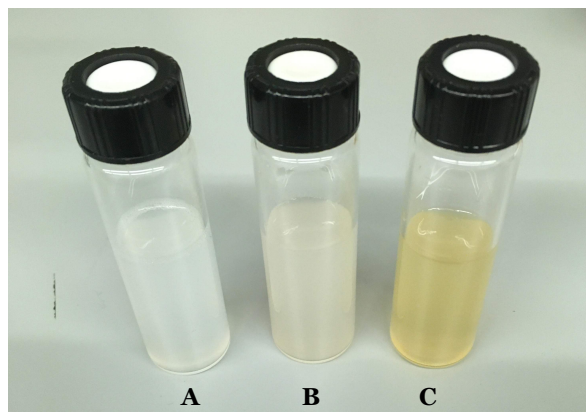


Figure 53. Nanoliposomes (A), *T. catigua* nanophytosomes (B) and *T. diffusa* nanophytosomes (C).

4.3.4.2. EE, particle size and surface charge

The UV-Vis spectra of *T. catigua* and *T. diffusa* nanophytosome formulations demonstrates a maximum absorption band at 280 nm (**Figure 54**), of which signal intensity was further used to calculate the EE.

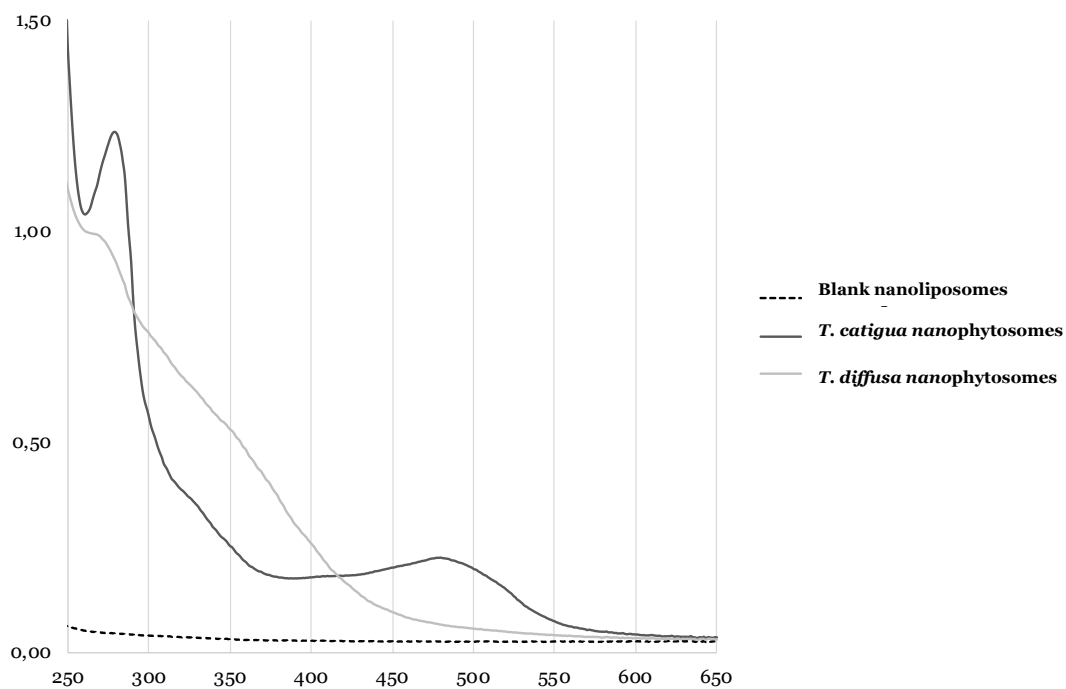


Figure 54. UV-Vis spectra of blank nanoliposomes, and *T. catigua* and *T. diffusa* nanophytosomes.

The EE, here assessed in terms of flavonoid content, is of 95% and 97% for *T. catigua* and *T. diffusa* nanophytosomes, respectively (**Table 14**). However, it is important to stress that *T. diffusa* nanophytosomes were prepared with a phospholipids:polyphenols molar ratio ten times higher than that of *T. catigua*. Therefore, the amount of polyphenols loaded in *T. catigua* nanophytosomes is also ten times lower. In fact, *T. catigua* nanophytosomes prepared with a higher relative abundance of polyphenols, the majority being flavan-3-ols and derivatives (**Figure 52A**, **Table 13**), exhibit low stability as evidenced by the presence of lipid fragments deposited at the bottom of the flask. This same phenomenon was already observed while studying the interaction of isolated flavan-3-ols with liposome systems (442). The authors described that the phospholipid bilayer of liposomes is sensitive to the incorporation of catechins, mainly to epicatechin gallate and epigallocatechin gallate, which disrupt the organization of the lipid bilayer when at high concentrations, thus promoting fusing and aggregations processes that culminate in the precipitation of the giant phenolic/lipid-based structures. In addition to the importance of a gallate moiety, other structural features, such as the stereoisomerism of the C-ring and the number of hydroxyl groups on the B-ring, deeply influence the affinity of flavan-3-ols for lipid bilayers along with the alteration of membrane structure and integrity (442). Therefore, the preparation of stable phytosome formulations with *T. catigua* is only possible by using lower amounts of its flavonoid-rich extract.

The nature of flavonoid-phospholipid interactions determines other physical-chemical properties of the vesicles, including size and surface charge (**Table 14**). The produced blank nanoliposomes have a mean particle size of 100 ± 11.3 nm and negative surface charge of -11.8 ± 0.6 mV. The nanophytosomes have size and surface charge dependent on the chemical composition of each flavonoid-rich extract (**Table 14**). When compared with blank nanoliposomes, *T. diffusa* nanophytosomes have similar size but a more negative surface charge (**Table 14**), suggesting that the entrapped flavonoids interact with phospholipids changing the number and/or organization of the water molecules that surround the phospholipid polar groups. The more negative Zeta potential values prevent the interactions with similar neighbour vesicles, avoiding the cluster formation and increasing the stability of the formulations (443).

Table 14. Mean particle size, polydispersity index (PDI), Zeta potential, and entrapment efficiency (EE) of blank nanoliposomes and *T. catigua* and *T. diffusa* nanophytosomes.

	Mean particle size (nm) ^a	PDI ^a	Zeta potential (mV) ^a	EE (%)
Blank nanoliposomes	104.4 ± 11.3	0.355 ± 0.005	-11.88 ± 0.60	
<i>T. catigua</i> nanophytosomes (97:3) ^b	74.4 ± 7.8	0.353 ± 0.007	-10.33 ± 1.85	95
<i>T. diffusa</i> nanophytosomes (70:30) ^b	118.3 ± 18.0	0.308 ± 0.004	-19.47 ± 1.04	97

^a Results represent the mean ± SD.^b Phospholipids:extract molar *ratio*.

On the other hand, *T. catigua* flavonoid-rich extract leads to the formation of phytosomes with lower diameter and a surface charge similar to that of blank nanoliposomes (**Table 14**). Therefore, the entrapment of *T. catigua* flavonoids results in vesicles with greater curvature (smallest diameter), without affecting the organization of the water molecules in the hydrophilic shells of the PL-bilayer. The lower amount of entrapped extract in *T. catigua* nanophytosomes may also contribute to these results.

The observed differences must result from the nature of interactions between the distinct flavonoids and phospholipids, due to the degree of affinity of flavonoids to the membrane bilayer, and their distinct localization and orientation within the phospholipid palisade. In fact, while comparing the effects of the loading of four distinct flavonoids on the attributes of lipid bilayers, other authors reported similar results, describing liposomes entrapping procyanidins (catechin and epicatechin oligomers) that were smaller in size and dispersed more uniformly than the ones entrapping apigenin and luteolin (438). They justified that procyanidins have relatively stronger lipophilicity than apigenin or luteolin and affect the extent of hydration of the phospholipid-water interface, easily binding to the hydrophobic tail of lecithin, thus favouring their location inside the lecithin bilayer. Furthermore, the lower relative water solubility and partition coefficient of apigenin and luteolin in the lipid bilayer favoured the bond of their hydroxyls to the polar heads of lecithin in the outside of the lipid bilayer, resulting in a larger particle size. These findings are also supported by the detailed description of the interactions between catechins and phospholipids in terms of hydrogen bonding, adsorption/absorption, molecular orientation, stereoisomerism, and presence of hydroxyl and galloyl functional groups among catechins (444).

The influence of the phenolic composition on the morphology of the phytosomes is likewise visible by TEM (**Figure 55**). All the nanostructures are spherically shaped particles but have demarked morphologic peculiarities. It is possible to observe that *T. diffusa* nanophytosomes (**Figure 55B**) are larger than the ones containing *T. catigua* (**Figure 55C**), as detected by DLS. Furthermore, *T. catigua* nanophytosomes are darker than the other two nanostructures. Therefore, the incorporation of flavan-3-ols and of their derivatives and oligomers present in *T. catigua* flavonoid-rich extract in the PL bilayer somehow increases the membrane affinity for uranyl-acetate 2%, enhances its contrasting effect, and even allows its more homogeneous dispersion in the lipid vesicle.

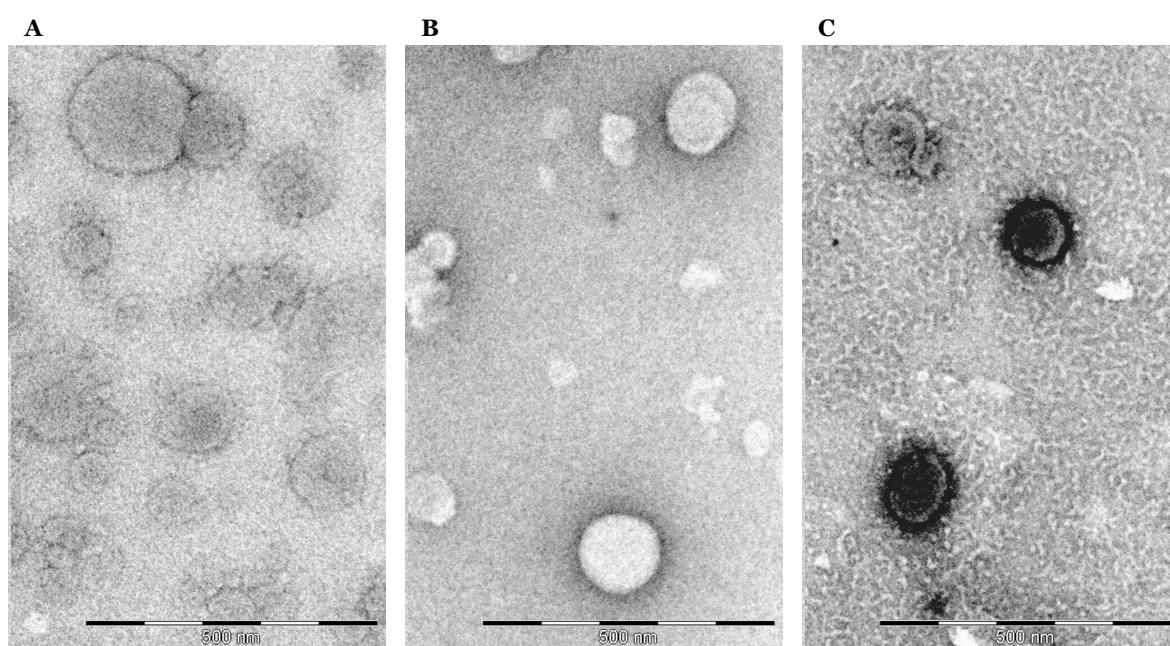


Figure 55. Morphology analyses by TEM of blank nanoliposomes (A), *T. diffusa* nanophytosomes (B), and *T. catigua* nanophytosomes (C).

4.3.4.3. Neuroprotective effects in cell models of induced neuroinflammation and neurotoxicity

In resemblance to the evaluation of the neuroprotective effects of *T. catigua* and *T. diffusa* crude aqueous extracts, and rutin-nanoliposomes, the performance of nanophytosomes was also tested in cell models of induced neuroinflammation and neurotoxicity.

The treatment of SH-SY5Y cells with *T. catigua* and *T. diffusa* nanophytosomes do not reduce cell viability (**Figure 56A** and **B**). The pre-treatment for 22 h with the nanophytosomes cannot protect cells from 6-OHDA/AA-induced toxicity (100 mM, dissolved in 0.02% AA solution), at least, when assessed in terms of cell viability by MTT

reduction (**Figure 56C and D**). Opposite results were obtained when cells are treated with *T. catigua* and *T. diffusa* crude extracts (**Figure 43**), thus the protective mechanism of the crude extracts may be mediated by activation of extracellular signalling pathways, such as MAPK, which may not occur when the extracts are entrapped in the lipid vesicles. Other hypothesis is that the intracellular amount of compounds released by the nanophytosomes, which is much lower when compared to that delivered in crude extracts, is not sufficient to display protective mechanisms.

On the other hand, the pre-treatment with *T. catigua* and *T. diffusa* nanophytosomes at their maximum tested concentrations protects SH-SY5Y cells from glutamate-induced toxicity (50 mM) (**Figure 56E and F**). At its highest tested concentration (phospholipids 225 μ M + extract 1.50 μ g/mL), *T. catigua* nanophytosomes hold cell viability around 78%. In a similar effect, the pre-treatment with *T. diffusa* nanophytosomes (phospholipids 113 μ M + extract 10.62 μ g/mL) holds cell viability at approximately 81%. Therefore, the protective effects of these phytosomes are observed on the micromolar range. Since opposite results were obtained when cells are treated with *T. catigua* and *T. diffusa* crude extracts (**Figure 43**), it may be that the formulations increase the release of the entrapped compounds and enhance their protective mechanisms.

The viability of BV-2 cells, determined by the MTT reduction assay, is not affected by cell treatment with *T. catigua* and *T. diffusa* nanophytosomes (**Figure 57A and B**), nor when cells are exposed to the extracts and IFN- γ (**Figure 57C and D**). When BV-2 cells are insulted with IFN- γ (20 U/mL) the NO levels raise up to approximately 65%. Only *T. diffusa* nanophytosomes are capable to reduce NO production with statistical significance, in a concentration-dependent effect (**Figure 57F**). The pre-treatment of BV-2 cells with the crude aqueous extract of *T. diffusa* also exerted the same protective effect (**Figure 46**), although the concentration of the extract was considerable higher than the one used in phytosomes. Therefore, by using the *T. diffusa* nanophytosome formulation the cell delivery of flavonoids may be enhanced leading to neuroprotective effects at lower extract concentrations. The *T. catigua* nanophytosome formulation fails to reduce NO production (**Figure 57E**), likewise observed for *T. catigua* crude extract. Once again, it is important to highlight that both nanoformulations share the same phospholipid molarity, thus their distinct performances can only result from their phenolic composition.

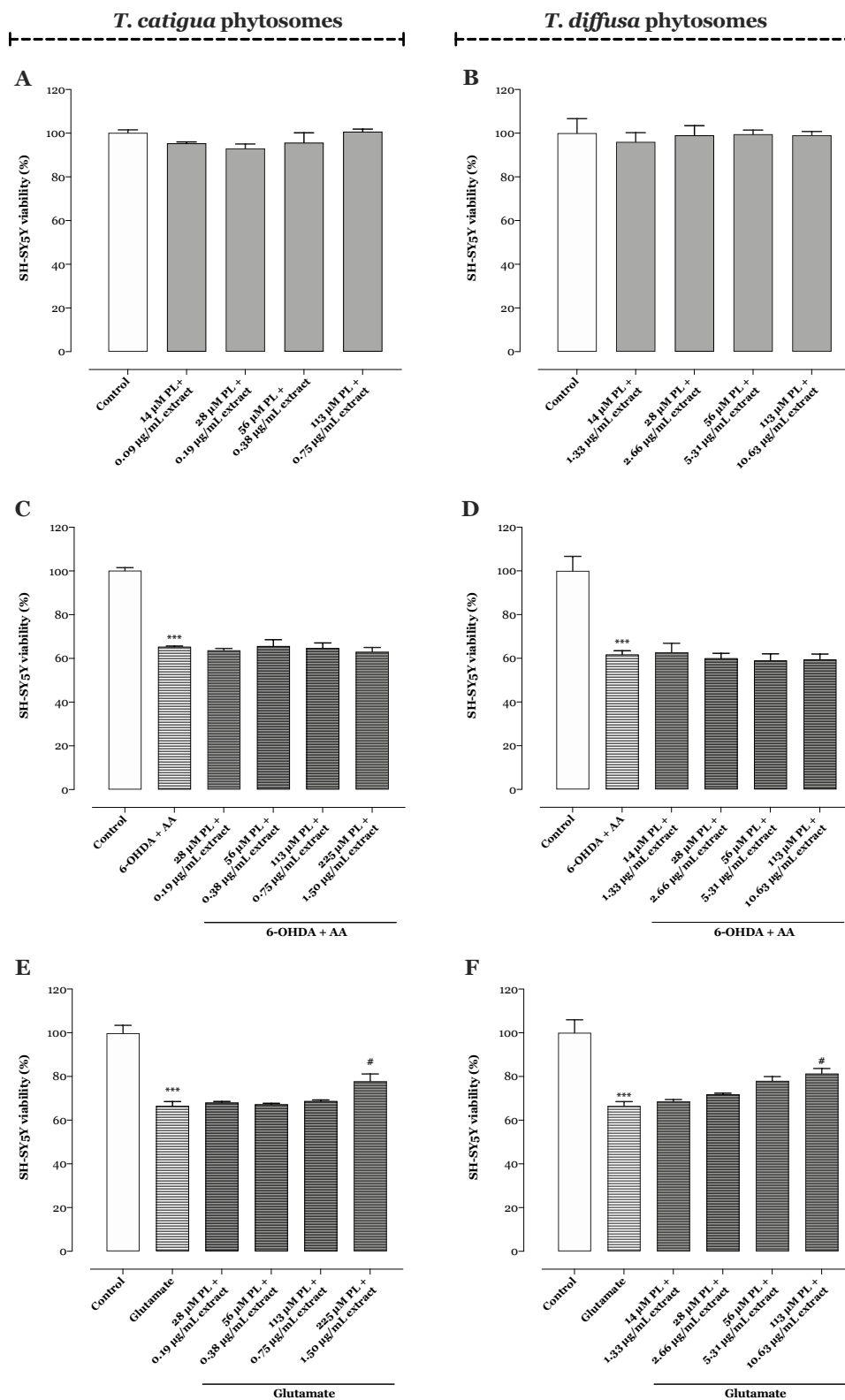


Figure 56. Neuroprotective effects of *T. catigua* and *T. diffusa* nanophytosomes in SH-SY5Y cells. Effects of formulations over cell viability assessed by MTT assay (**A** and **B**). Effects of the pre-treatment in cells co-exposed to 6-hydroxydopamine/ascorbic acid-induced toxicity (6-OHDA/AA; 100 mM + 0.02 %) (**C** and **D**). Effects of the pre-treatment in cells co-exposed to glutamate (50 mM) (**E** and **F**). The results are expressed as mean $\pm$ SEM from five independent assays, each performed in triplicate. The statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* (level of statistical significance * p < 0.05). ***, significantly different from the control with p < 0.001; #, significantly different from cells exposed to the toxicants with p < 0.05.

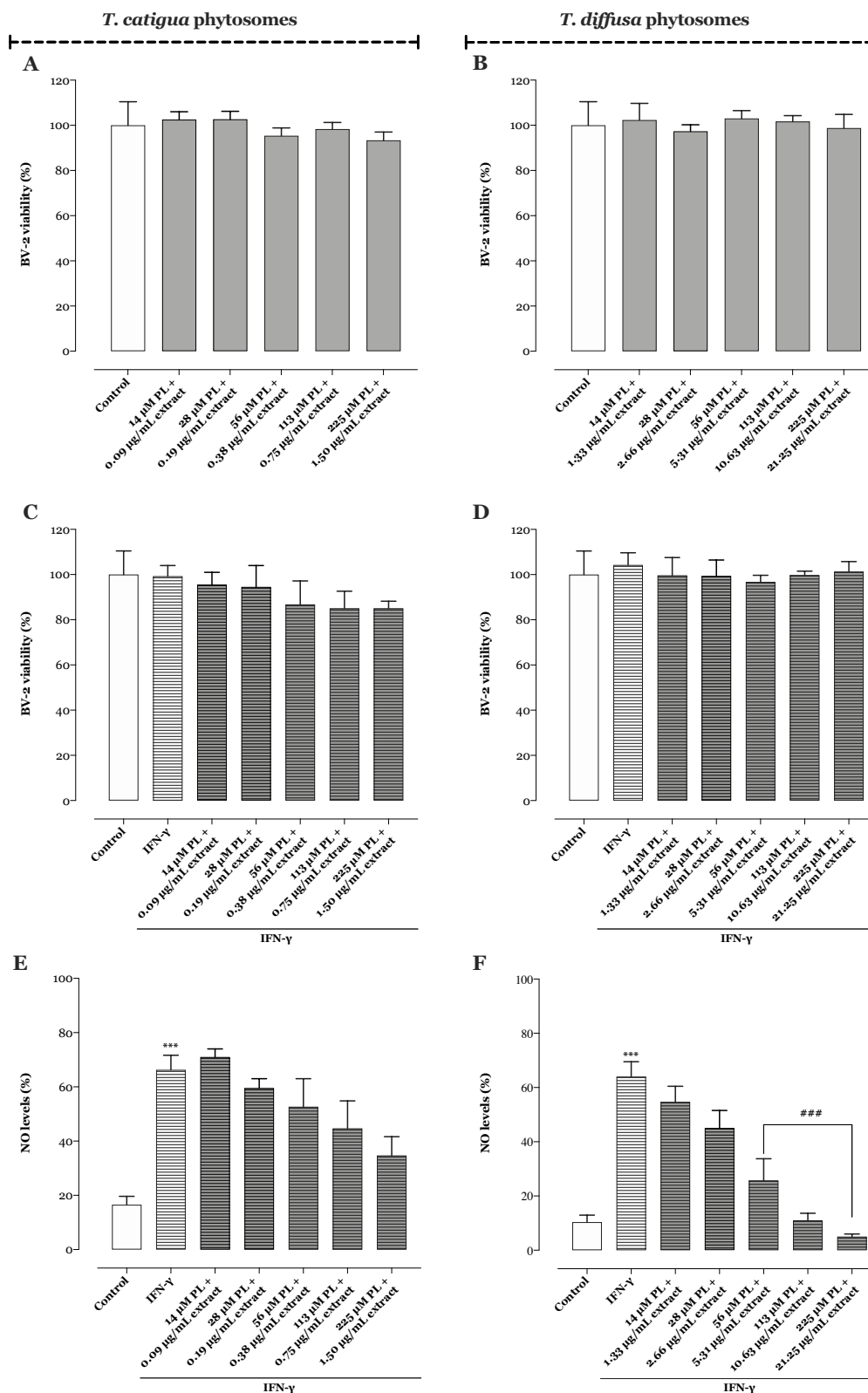


Figure 57. Performance of *T. catigua* and *T. diffusa* nanophytosomes in BV-2 microglia cells. Viability of BV-2 cells assessed by MTT (**A**, **B**, **C** and **D**). Quantification of NO levels in culture supernatants by Griess reaction (**E** and **F**). Results obtained from untreated cells (control), from cells after insult with IFN- γ (20 U/mL), and from cells pre-treated with *T. catigua* and *T. diffusa* nanophytosomes followed by IFN- γ insult (20 U/mL). The results were expressed as mean $\pm$ SEM from five independent assays, each performed in triplicate. The statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* (level of statistical significance * $p < 0.05$). ***, significantly different from the control with $p < 0.001$; # and ###, significantly different from the cells exposed to IFN- γ with $p < 0.05$ and $p < 0.001$, respectively.

Overall, the results of this study indicate that by the conjugation of phospholipids with flavonoid-rich extracts obtained from *T. catigua* and *T. diffusa* species it is possible to produce functional lipid-based nanoformulations. The entrapment of the flavonoid-rich extracts results in new nanostructures. The nature of the phenolic content deeply affects the phospholipid-phenolics interactions and determines the re-arrangement of the complexes, even though *T. catigua* and *T. diffusa* nanophytosomes demonstrated features suitable for formulations intended to reach and act in the CNS.

4.3.4.4. Comparative study of the stability profiles

LUMiSizer® consists of an analytical photocentrifuge, which makes use of the Space and Time resolved Extinction Profile Technology, known as STEP-Technology® (L.U.M GmbH, Berlin, Germany). This technology calculates the intensity of transmittance across the entire cell, as a function of time and radial position. Thus, it is used to study suspensions demixing behaviour and stability, and to obtain particle size distributions by the estimation of the particle migration velocity. Such instrumentation draws on the capacity of particles in a suspension to change the transmittance through the sample in which they are suspended. The combination of this characteristic with the centrifugal force, capable of accelerating particle migration, allows the acquisition of a transmission profile, also designated as transmission fingerprint (444). In particular, LUMiSizer® records the progression of the transmission profiles over time and space, providing information concerning to the kinetics of concentration changes due to instability phenomena, including creaming, sedimentation, flocculation, coalescence or phase separation. This technique not only allows the qualitative *in situ* analysis of the separation behaviour of the analysed samples granted by the transmission profiles, as it also provides quantitative data by the determination of the instability index, as well as the migration velocity determination by the particle size distribution analysis (445).

With this methodology we intended to study the stability profile of *T. catigua* and *T. diffusa* nanophytosomes. Furthermore, to understand if the introduction of mannitol as cryoprotectant in the dialysis step, with subsequent lyophilisation of the formulations, would allow to obtain more stable lyophilised formulations, which may provide a more advantageous pharmaceutical dosage form. To that purpose, new formulations were prepared at 5 mM phospholipid while keeping the same previous phospholipids:phenolics *ratios*. Then, aliquots were dialysed with suspension buffer, whilst others were dialysed with suspension buffer supplemented with mannitol, and then lyophilized (**Figure 58**).

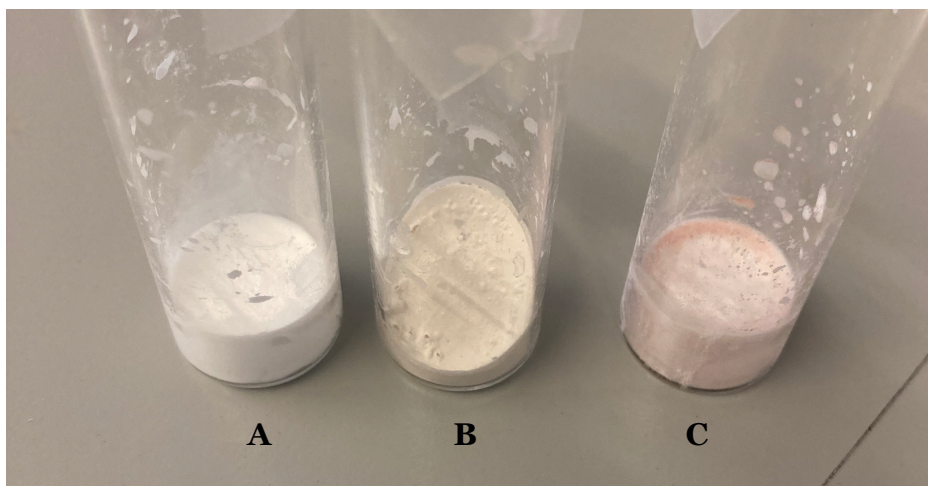


Figure 58. Lyophilized formulations of nanoliposomes (A), *T. diffusa* nanophytosomes (B), and *T. catigua* nanophytosomes (C).

The calculated EE is of 96% for *T. diffusa* nanophytosomes and of 95% for *T. catigua* nanophytosomes (**Table 15**). On the other hand, a similar efficiency was reported for the lyophilized *T. diffusa* phytosomes, while it decreased to 81% in lyophilized *T. catigua* nanophytosomes (**Table 15**).

The mean particle size, PDI, and Zeta potential of these formulations are displayed in **Table 15**. In general, the formulations prepared with 5 mM phospholipid display higher mean particle sizes than the corresponding ones prepared with 900 μ M (**Table 14**). However, the relative effects of the extracts upon the physicochemical characteristics of the lipid vesicles follows the same patterns as before. In more detail, the entrapment of the *T. diffusa* extract results in vesicles with a mean particle size closer to that of the blank nanoliposomes, while *T. catigua* phytosomes display the lowest value of the three formulations (**Table 15**).

Moreover, *T. catigua* and *T. diffusa* phytosomes display more negative Zeta potential values than the blank nanoliposomes (**Table 15**), in accordance with the previous findings (**Table 14**).

Table 15. Mean particle size, polydispersity index (PDI), Zeta potential, and entrapment efficiency (EE) of blank nanoliposomes, and *T. catigua* and *T. diffusa* lyophilized nanophytosomes, all prepared at 5 mM of phospholipid.

	Mean particle size (nm) ^a	PDI ^a	Zeta potential (mV) ^a	EE (%)
Blank nanoliposomes	209.0 ± 44.9	0.330 ± 0.024	-5.90 ± 1.08	
<i>T. catigua</i> nanophytosomes (97:3) ^b	179.9 ± 2.4	0.319 ± 0.050	-11.35 ± 2.49	95
<i>T. diffusa</i> nanophytosomes (70:30) ^b	187.2 ± 10.1	0.300 ± 0.011	-16.56 ± 1.91	96
Lyophilized formulations				
Blank nanoliposomes	87.5 ± 6.9	0.324 ± 0.076	-7.07 ± 2.25	
<i>T. catigua</i> nanophytosomes (97:3) ^b	165.4 ± 5.5	0.254 ± 0.050	-13.55 ± 0.99	81
<i>T. diffusa</i> nanophytosomes (70:30) ^b	160 ± 13.7	0.190 ± 0.036	-18.98 ± 1.96	95

^a Results represent the mean ± SD from three independent formulations.

^b Phospholipids:phenolics molar ratio.

4.3.4.4.1. Transmission profiles

The transmission profiles represent a time-resolved motion of the particles in the cell during the centrifugation process, leading to the appearance of a clarification profile, which is characterized by an increase in transmission, from the top of the cell (left) to the bottom (right), due to phase separation.

The transmission profiles for all tested formulations, as well as the corresponding sample cells after the accelerated stability and demixing behaviour assay using LUMiSizer®, are depicted in **Figure 59** and **Figure 60**, respectively. The first profiles are displayed in red and the latest in green. Except for *T. catigua* lyophilized nanophytosomes (**Figure 59D**), which exhibit some characteristics of a creaming profile, all the other nanoformulations showed typical sedimentation profiles (**Figure 59A, B, C and E**).

Besides the visual aspect of colloidal suspensions after centrifugation (**Figure 60**), it was also possible to observe that either blank nanoliposomes or *T. catigua* and *T. diffusa* nanophytosomes exhibit distinct colours, which is in line with their composition, namely, colourless (transparent), light pink and light yellow, respectively (**Figure 58**).

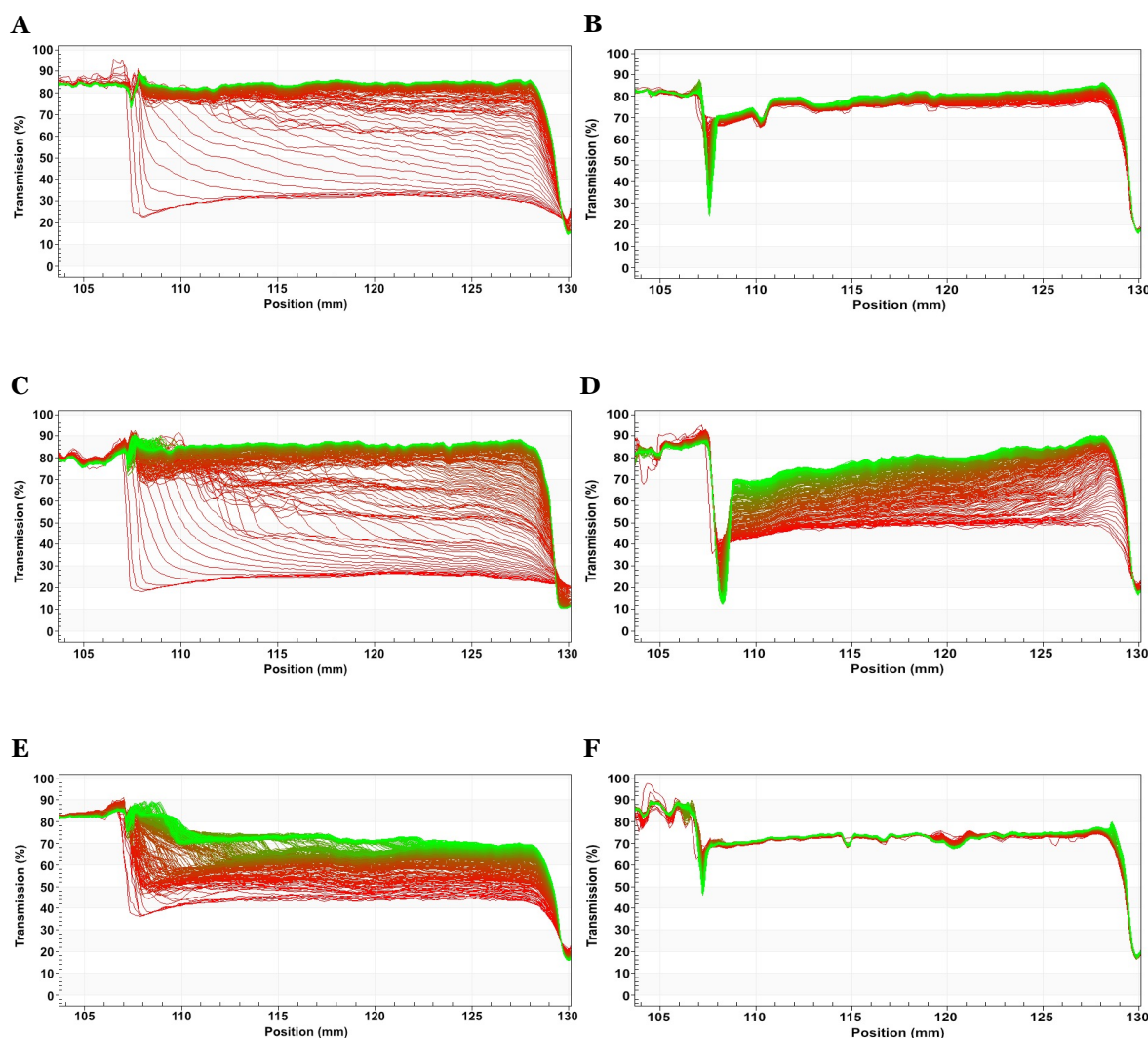


Figure 59. Evolution of the transmission profiles of the formulations. The transmission profiles of blank nanoliposomes (**A**), *T. catigua* nanophytosomes (**C**) and *T. diffusa* nanophytosomes (**E**) are recorded by analysing the lipid suspensions after dialysis. The transmission profiles (**B**), (**D**) and (**F**) are from the respective formulations when dialyzed with 20% mannitol suspension buffer, then lyophilized, and finally re-hydrated prior to analysis. The colour of the transmission profiles underwent changing from red to green as the time of centrifugation progressed from 0 to 29 min. X-axis represents the position of cell, having the rotor as reference, and Y-axis represents the percentage of light transmission.

In a more detailed data analysis, the transmission profile of blank nanoliposomes (**Figure 59A**) is typical of a polydisperse suspension and suggests the existence of a population of larger particles, since the first profiles are more separated between each other than the remaining ones (446). Then, with time, the remaining fractions of particles seem to have a more uniform size distribution, given that the distance between the profiles becomes smaller and more constant. In fact, a small sediment is visible in the bottom of the sample cell (**Figure 60A**), which corroborates the previous conclusion of the existence of a heterogeneous particle population that leads to sedimentation. Furthermore, these

findings agree with the PDI of 0.330 ± 0.024 determined by DLS for blank nanoliposomes (**Table 15**), which also indicates that are present multiple particle size populations. The PDI can range from 0.0, in a perfectly uniform formulation in terms of particle size, to 1.0 in a highly polydisperse formulation with multiple particle size populations. In lipid-based carriers, such as nanoliposomes, a PDI of 0.3 and below is acceptable and indicates a homogenous population of lipid vesicles (447).

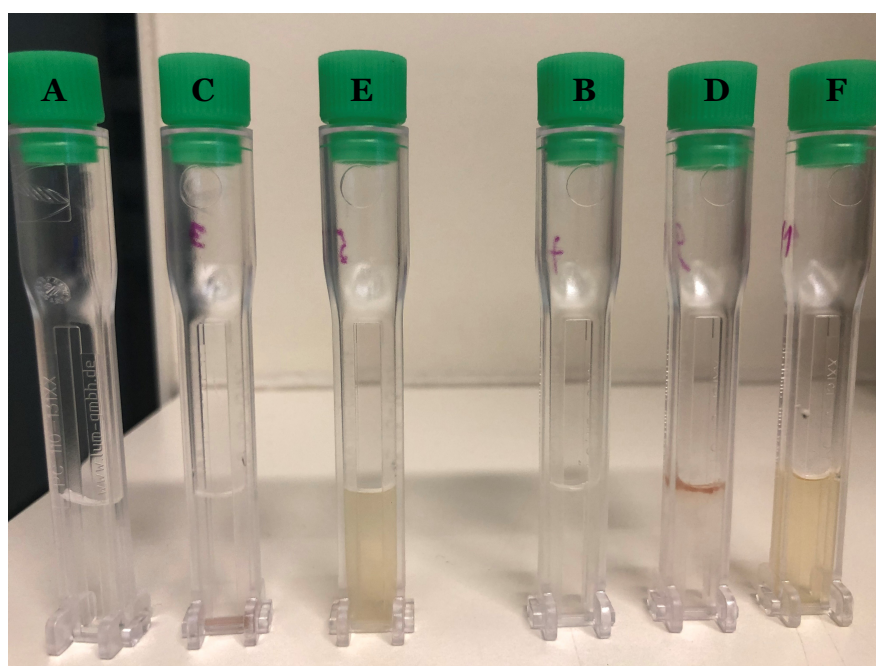


Figure 60. Polycarbonate photocentrifuge cells with the lipid formulations after the accelerated stability and demixing behaviour assay using LUMiSizer®. (A) Blank nanoliposomes; (C) *T. catigua* nanophytosomes; (E) *T. diffusa* nanophytosomes; (B), (D) and (F) are the respective formulations when dialyzed with 20% mannitol suspension buffer, followed by lyophilization, and re-hydrated prior to analysis.

The dialysis with mannitol followed by lyophilisation seems to increase the stability of blank nanoliposomes, as no big changes in transmission occur during the centrifugation process (**Figure 59B**). In fact, no sediment is visible in the cell containing the lyophilized blank nanoliposomes after the assay (**Figure 60B**). In line with this observation, the lyophilized blank nanoliposomes present a smaller particle size than the original formulation, together with reduced PDI value and a more negative Zeta potential (**Table 14** and **Table 15**).

Regarding *T. catigua* nanophytosomes, the transmission profile is similar to that of blank nanoliposomes (**Figure 59A**), as depicted by the existence of a polydisperse colloidal suspension (**Figure 59C**). In line with these results, in the DLS analysis of *T. catigua*

nanophytosomes is obtained a PDI close to that found for blank nanoliposomes (**Table 15**). *T. catigua* lyophilized nanophytosomes displays a different transmission profile (**Figure 59D**). When compared with the respective non-lyophilized formulation, the more uniform separation profile suggests the existence of a more homogeneous population in terms of particle size distribution. This conclusion is also supported by the observation that the separation profiles are uniformly separated from each other over time. Thereby, *T. catigua* lyophilized nanophytosomes appear to be relatively stable. Besides, the separation process begins from the bottom to the top of the cell suggesting that the particles are migrating to the top of the cell during the centrifugation-imposed stress, which is similar to a creaming demixing process (corresponding to the migration of the dispersed phase of an emulsion) (448). Furthermore, the occurrence of a creaming mechanism is also physically observed by the appearance of a marked pink layer in the top of the photocentrifuge cell (**Figure 60D**). It substantiates such destabilization over time for *T. catigua* lyophilized nanophytosomes contrary to the light pink sediment formation in the bottom of the cell, which is characteristic of a sedimentation observed for the non-lyophilized *T. catigua* nanophytosomes (**Figure 60C**).

In its turn, *T. diffusa* nanophytosomes show a uniform separation profile (**Figure 59E**), which suggests the existence of a more homogeneous population in terms of particle size. In agreement, the separation profiles are uniformly separated from each other over time. Furthermore, with a PDI of 0.300 ± 0.011 , this formulation has the lowest value of all three non-lyophilized formulations (**Table 15**). Thus, this formulation appears to be the one more relatively stable. Such evidence is also supported by the absence of a visible sediment after centrifugation in the cell (**Figure 60E**). The profile of *T. diffusa* lyophilized nanophytosomes (**Figure 59F**) demonstrates that, just as observed for blank nanoliposomes and lyophilized blank liposomes (**Figure 59A and B**), the lyophilisation seems to enhance the stability of the colloidal system, as no large changes occur during the centrifugation process. In fact, no sediment is visible in the photocentrifuge cell (**Figure 60F**), and this formulation presents the lowest PDI value of all the tested formulations (**Table 15**).

4.3.4.4.2. Sedimentation velocities

The analysis of the particles by TEM indicated a close to spherical shape (**Figure 55**). Such evidence allows the determination of the velocity of migration (v_m), by using the equation $v_m = k \cdot r^2$, where k is a constant and r is the radius of the particle. In this way,

this equation shows that the migration velocity has a quadratic relation with the particle size.

The sedimentation velocity distributions for the tested formulations are depicted in **Figure 61**. The sedimentation velocity distributions of the formulations clearly reveal the presence of populations of distinct particle size, even in the same order of magnitude, as also demonstrated by the median (d_{50}) sedimentation velocity values (**Table 16**).

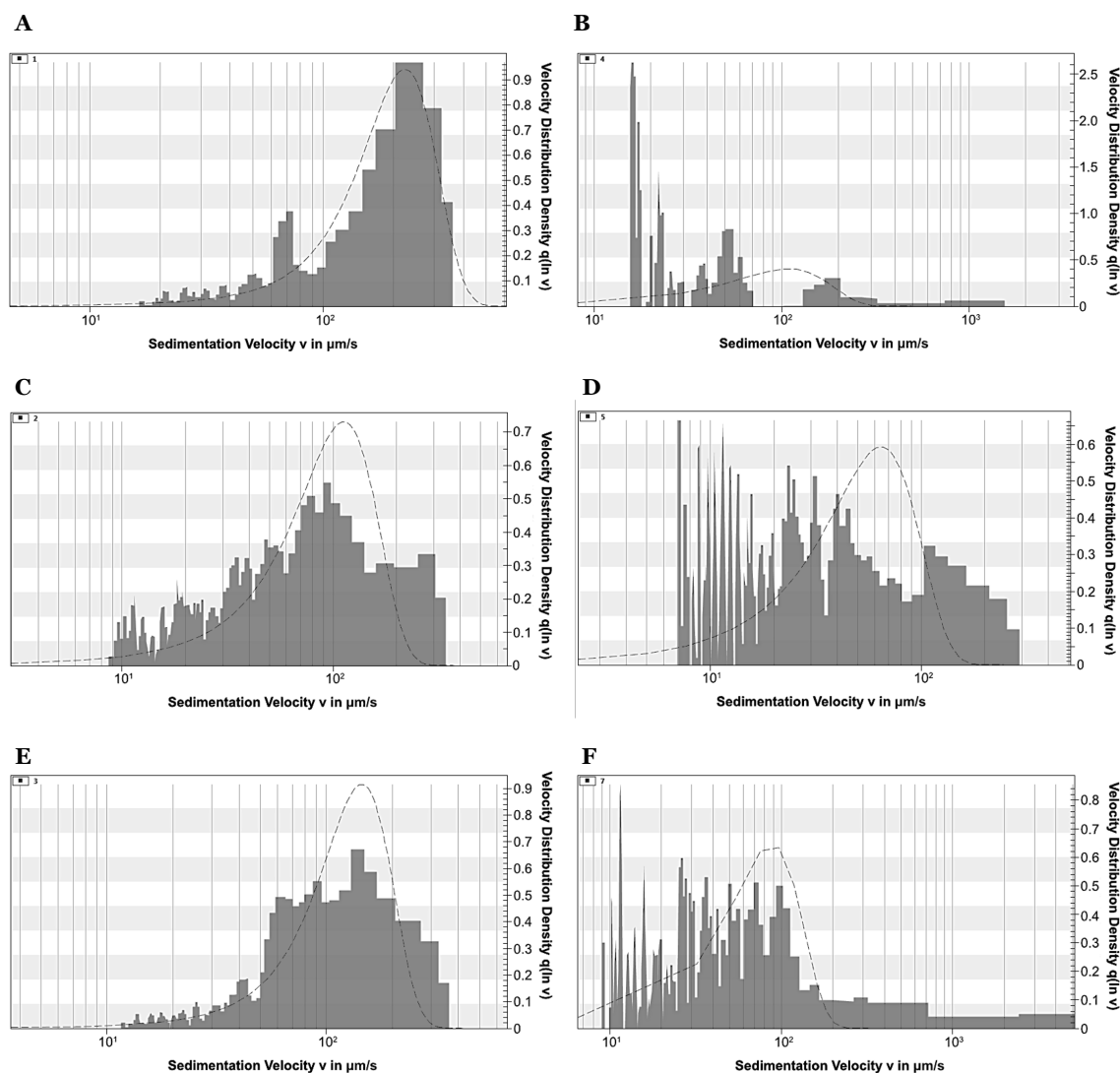


Figure 61. Distribution of the sedimentation velocity of the nanoformulations: (A) blank nanoliposomes; (C) *T. catigua* nanophytosomes; (E) *T. diffusa* nanophytosomes; (B), (D) and (F) are the respective formulations when dialysed with 20% mannitol suspension buffer, then lyophilized, and finally re-hydrated prior to analysis.

It is possible to observe that a global tendency for smaller particle size (reflected in the reduction of the sedimentation velocity) occurs in the lyophilized formulations. It has significant relevance when compared blank nanoliposomes (**Figure 61A**) and *T. diffusa*

nanophytosomes (**Figure 61C**) with their corresponding lyophilized versions (**Figure 61B** and **E**, respectively).

This is also expressed in terms of the sedimentation velocity distribution. Most particles of the non-lyophilized formulations evidence higher sedimentation velocities (superior to 10^2), while in the lyophilized ones the distributions are slightly displaced to inferior values, even in the same order of magnitude, as also revealed by the average sedimentation velocity values (**Table 16**). The lowest average sedimentation velocities are observed for *T. diffusa* formulations, although they do not show a significant variation before and after lyophilization (**Table 16**). However, a significant variation is verified between the blank liposome formulations, and between *T. catigua* formulations (**Table 16**).

Table 16. Average sedimentation velocity and instability index of the formulations.

Formulations	Sedimentation velocity (d50) ($\mu\text{m/s}$)	Instability index
Blank nanoliposomes	204.3	0.910
Lyophilized blank nanoliposomes	53.54	0.403
<i>T. catigua</i> nanophytosomes	116.4	0.947
Lyophilized <i>T. catigua</i> nanophytosomes	61.11	0.719
<i>T. diffusa</i> nanophytosomes	32.41	0.615
Lyophilized <i>T. diffusa</i> nanophytosomes	39.55	0.085

4.3.4.4.3. Instability indexes

The separation analysis of a suspension under accelerated conditions also enables the calculation of the instability index, which allows a fast stability ranking and even, when possible, an estimation of the shelf-life. The instability index calculation is based on the clarification process, at a given separation time, divided by the maximum clarification. The dimensionless instability index value ranges from 0 (more stable suspension) to 1 (more unstable suspension). This indicates that samples with higher clarification rates tend to be more unstable, and attributes to this parameter a high degree of value in the detection of potentially unstable formulations (449).

T. diffusa lyophilized nanophytosomes present the lowest instability index (**Table 16**), suggesting that, physically, this is a very stable formulation when compared with the other tested ones. In fact, *T. diffusa* nanophytosomes also display the most negative Zeta potential among the tested formulations (**Table 15**). Once more, the results also suggest that a stabilization effect is promoted when the formulation are dialyzed with mannitol and lyophilised, since for all group samples (non-lyophilized *versus* lyophilized) the instability index is substantially reduced for lyophilized samples in relation to the corresponding colloidal suspensions (**Table 16**). Therefore, either mannitol or lyophilisation, or both processes, clearly interfere in the physical stability of the formulations since is observed a modification of the separation behaviours. These processes seem to stabilize blank nanoliposomes and *T. diffusa* nanophytosomes. In the case of *T. catigua* nanophytosomes, besides their physical stabilization, they seem to interfere at the chemical level since the density characteristics are also altered. These results also emphasize differences regarding the impact of the nature of the flavonoid-rich extracts in the physical behaviour of the formulations suggesting that the incorporation of *T. diffusa* extract could lead to a higher physical stabilization in comparison to *T. catigua* extract. Such results are in line with the differences also found in terms of transmission profile (**Figure 59**), and possibly result from the distinct phenolic profile of the two species (**Figure 52**) and corresponding interactions between the phenolic compounds and the phospholipids supporting the lipid vesicles.

According to the accelerated stability and demixing behaviour results, it can be assumed that both the entrapment of the extracts, and the dialysis with mannitol followed by lyophilisation, improve the physical stabilization of the formulation. It is well known that long-term stability of liposomes by lyophilisation can be achieved if cryoprotectants are added to their structure to avoid the mechanical stress caused by ice crystal formation during freezing (450). Cryoprotectants, such as mannitol, postpone these degrading mechanisms because they do not crystallize, rather form an amorphous frozen matrix upon cooling, and prevent both fusion of the particles and crystal formation during freezing due to the interactions with the headgroups of the phospholipid counteracting fusion or membrane disruption (451).

CHAPTER IV

Conclusions

5. Conclusions

In a general overview, the work conducted in this research aimed to explore the neuroprotective potential of natural compounds from medicinal plant extracts and use the extracts towards the development of neuroactive nanoformulations. Based on the results some conclusions can be draw:

- Flavonoids were identified as the major sub-class of phenolic compounds in *T. catigua* stem bark and *T. diffusa* leave crude aqueous extracts. In traditional medicine, the stem bark of *T. catigua* is used to prepare hydro-alcoholic decoctions and the leaves of *T. diffusa* are used to prepare infusions and decoctions. Hence, flavonoids are likely to be present in those preparations.
- The *in vitro* screening demonstrated that *T. catigua* and *T. diffusa* crude aqueous extracts can interact with multiple targets and pathways underpinning neurodegeneration, as assessed by their anti-radial activity, the inhibition of several enzymes, antiglycation activity, and by their protective effects in cell models of induced neuroinflammation and cytotoxicity. The neuroprotective effects of the extracts must be related to their chemical composition. Furthermore, flavonoids are most likely responsible, at least in part, for the neuroactive properties linked to the use of these species in folk medicine.
- The qualitative and quantitative phenolic profiles of the extracts have impact on their biological activities. *T. catigua* crude aqueous extract was characterized as a rich mixture of flavan-3-ols, their oligomers and phenylpropanoid substituted derivates, while *T. diffusa* extract was dominated by luteolin derivatives. The higher phenolic content found for *T. catigua* may explain why it exceled in all cell-free assays. However, only *T. diffusa* extract could protect BV-2 cells from INF- γ pro-inflammatory insult, reflected by the reduced production of NO.
- The outlined extraction procedure allowed to obtain phospholipids from egg yolk by using only ethanol and acetone, thus it follows the European guidelines for the utilization of organic solvents in the production of food products. This procedure aggregates valuable features for a possible industrial scale-up. In addition, the main-by products (cholesterol and carotenoids) are obtained in separated acetone solutions, thus allowing their recovery and further applications.

- The phospholipid-rich fraction proved to be suitable for the development of flavonoid lipid-based nanocarriers. Their combination with rutin resulted in nanoliposomes with high entrapment efficiency and structural features adequate to the further development of bioactive formulations. The neuroprotective effects of rutin-nanoliposomes against glutamate toxicity induced in SH-SY5Y cells suggest that this formulation should be studied in pre-clinical models of neurodegenerative disorders to fully understand its potential.
- The combination of phospholipid-rich fractions with flavonoid-rich extracts obtained from solid-phased extraction of *T. catigua* and *T. diffusa* crude extracts, resulted in nanophytosomes.
- The biological activities of the nanophytosomes were determined by the distinct phenolic content of the plant extracts. In some cases, the phyto-phospholipid complexes enhanced the bioactivity of the extracts, whilst in other situations the inverse was also observed. These new formulations have an identity of their own and exhibited neuroprotective effects within the micromolar range.
- The entrapment of the flavonoid-rich extracts in lipid vesicles seemed to tend to stabilize the nanostructure. In addition, the nanophytosome dialyses with mannitol followed by lyophilization improved the long-term stability of the formulations. The utilization of lyophilized formulations, rather than the fresh prepared ones, may be of great value since it not only may favour new administration routes, as it may provide a product with extended shelf-life.
- It is important to highlight that Pharmacognosy is still a valid and upward science, especially when it is merged with the most recent advances in drug development, such as nanotechnology.

CHAPTER V

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6. References

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