Mentha pulegium Linnaeus:

from chemistry to cell protection against _t_-BHP-induced toxicity

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in Analytical, Clinical and Forensic Toxicology

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<u>In loving memory of my grandmother Raquel Fernandes</u>

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ABSTRACT

Herbal teas are widely consumed for their valuable content on phytochemicals with antioxidant properties. This work represents the first attempt to establish a linkage between the chemical composition of *Mentha pulegium* L. infusion and its potential to prevent oxidative stress in cells. The phenolic profile was established by HPLC-DAD-ESI/MS^n, 12 out of 15 compounds being identified for the first time in this species. The infusion presented a total phenolic content of 122.92 mg/g (lyophilized extract), also demonstrated a remarkable antiradical activity against DPPH$^{\cdot}$ (EC_{50}= 39 µg/mL), $O_2^{\cdot -}$ (EC_{50}= 23 µg/mL) and $^{\cdot}NO$ (EC_{50}= 226 µg/mL) radicals. It also revealed to be very effective in protecting AGS and Caco-2 cell lines against *tert*-butylhydroperoxide induced toxicity as evaluated by the MTT assay, under a pre-treatment experimental model. Reduced glutathione detoxification mechanism was demonstrated to be involved in Caco-2 cells resistance, while the same was not observed for AGS cells. The presence of phenolic compounds inside the cells, after infusion removal, was confirmed by DPBA phenol fluorescence dye staining. A direct antioxidant effect can contribute to the observed protective effect. Attending to all the results here presented, the consumption of *M. pulegium* infusion provides bioactive compounds with great importance to the maintenance of a proper antioxidant imbalance in gastrointestinal cells.

KEYWORDS: *Mentha pulegium* L.; infusion; phenolic compounds; antioxidant effect; *tert*-butylhydroperoxide;

RESUMO

As plantas medicinais são amplamente consumidas, uma vez que representam uma vasta fonte de compostos com ação antioxidante. Neste trabalho foi explorada pela primeira vez a ligação entre a composição química da espécie *Mentha pulegium* L. e a capacidade para prevenir o stress oxidativo celular. O perfil fenólico foi estabelecido por HPLC-DAD-ESI/MS^n e 12 dos 15 compostos identificados foram descritos pela primeira vez nesta espécie. A infusão apresentou um teor fenólico total de 122.92 mg/g (extrato liofilizado) e demonstrou grande atividade contra os radicais $DPPH^\cdot$ (EC_{50}= 39 µg/mL), $O_2^{\cdot -}$ (EC_{50}= 23 µg/mL) e $^\cdot NO$ (EC_{50}= 226 µg/mL). O pré-tratamento de células AGS e Caco-2 com a infusão, seguido de exposição ao *terc*-butil-hidroperóxido, revelou a sua eficiência na proteção contra a toxicidade induzida pelo agente agressor, aferida pelo ensaio de redução do MTT. A destoxificação pela glutationa reduzida demonstrou ser determinante para a resistência verificada nas células Caco-2, não se observando o mesmo relativamente às células AGS. Recorrendo à marcação por fluorescência de compostos fenólicos com DPBA foi confirmado que estes se encontravam dentro das células aquando da exposição ao agente agressor, sugerindo a contribuição de um efeito direto na proteção mediada pela infusão. Considerando todos os resultados aqui apresentados pode-se concluir que a ingestão da infusão de *M. pulegium* fornece compostos bioativos com particular interesse para a manutenção do equilíbrio antioxidante de células gastrointestinais.

PALAVRAS-CHAVE: *Mentha pulegium* L.; infusão; compostos fenólicos; efeito antioxidante; *terc*-butil-hidroperóxido;

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ABREVIATIONS AND SYMBOLS

AChE Acetylcholinesterase

ATP Adenosine triphosphate

CAT Catalase

DAD Diode-array detector

DNA Deoxyribonucleic acid

EC_{50} Half maximal effective concentration

ESI Electrospray ionization

GSH Glutathione reduced form

GSx Total glutathione

GSSG Oxidized glutathione

HPLC High-performance liquid chromatography

LDH Lactate dehydrogenase

m/z Mass-to-charge *ratio*

MS Mass spectrometry

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ROS Reactive oxygen species

RNS Reactive nitrogen species

SOD Superoxide dismutase

Chapter I - Introduction

1. Phytotherapy

Medicinal plants are used as pharmacotherapy tools worldwide. Since remote times, different species were selected and applied to prevention and treatment of diverse pathologies. This ancient practice has been replaced by modern medicine brought with the 20th century. But it is a fact that for approximately 80% of world's population, phytotherapy constitutes the first line of treatment, often being the only one existing. Moreover, in 1978 the World Health Organization recognized Traditional Medicine as a valid form of primary healthcare, being continuously working towards its regulation and correct use (1).

Nature is a huge source of chemical compounds with pharmacologic and toxic activities, also having a key role in the development of novel synthetic drugs. In the past thirty years, major contributions have been made to diverse fields, such as neurodegenerative diseases, cancer treatment and anti-inflammatory agents (2).

A revivalist trend as recently brought back phytotherapy to the spotlight. All over Europe this market has shown a big expansion, particularly in Germany, France and Italy (3). New approaches to Traditional Medicine, based on reverse-pharmacology, seem to allow the perfect linkage between popular knowledge, science and the positive health outcomes for consumers (4). Moreover the easy access, low prices, strong marketing and the wrong popular idea of absence of associated risks are the main boosters behind this consumption's growth. These products are presented in pharmaceutical dosage forms like capsules, tablets and drops. More traditional forms can also be found at herbal stores (5). Medicinal plants are currently applied to cure and prevent respiratory, gastrointestinal and sleep disorders, to alleviate side effects of anticancer therapies, to fight depression, to enhance weight loss and dietary supplementation, in dermatology and cosmetics, among other uses (6).

Such impact on public health justifies, *per se*, the need to understand and study, not only the chemical composition of these products, but also the risks and benefits resulting from their consumption. The increasing number of reports on interactions between herbal medicines and prescribed drugs reflects this reality. Additionally, adulteration of final products, misidentification of species, heavy metals contamination and the presence of pesticides are safety issues that must be taken into consideration (7). These events are complex to elucidate and have the disadvantage of both qualitative and quantitative variation of the chemical profile of the natural species, due to different growing conditions

and industrial and storage processes (8). For all the reasons presented above the pharmacological and toxic potentials of medicinal plants is an important and vast field for scientists to explore.

2. Cellular oxidative stress

A homeostatic cell environment is necessary to maintain proper physiological functions. A vast number of free radicals, resulting from both endogenous processes and exogenous sources, is responsible for the significant alteration of cellular homeostasis, leading to oxidative stress. Nevertheless, their physiological roles cannot be underestimated (9). As examples, reactive oxygen species (ROS) include either oxygen radicals like superoxide ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$), and peroxyl ($RO_2^{\cdot}$) radicals, or non-radical oxidizing agents, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), easily converted into radicals. Also, reactive nitrogen species (RNS) and reactive sulfur species (RSS), which are formed by thiols' reaction with ROS, play an active role in disrupting the imbalance between pro-oxidants and antioxidants.

The interest on these radical species has been increasing, since they are thought to be involved in different pathophysiological mechanisms behind neurodegenerative and cardiovascular diseases, diabetes, arthritis, osteoporosis and cancer (10).

The cellular damage depends on the doses and time of ROS exposure, triggering different pathways and modulating the action of enzymes and transcription factors. Diverse effects can be observed, from cellular proliferation by mitogenic stimulation in low doses, to cellular cycle arresting or even cellular death by apoptosis or necrosis when exposed to high doses (Fig. 1).

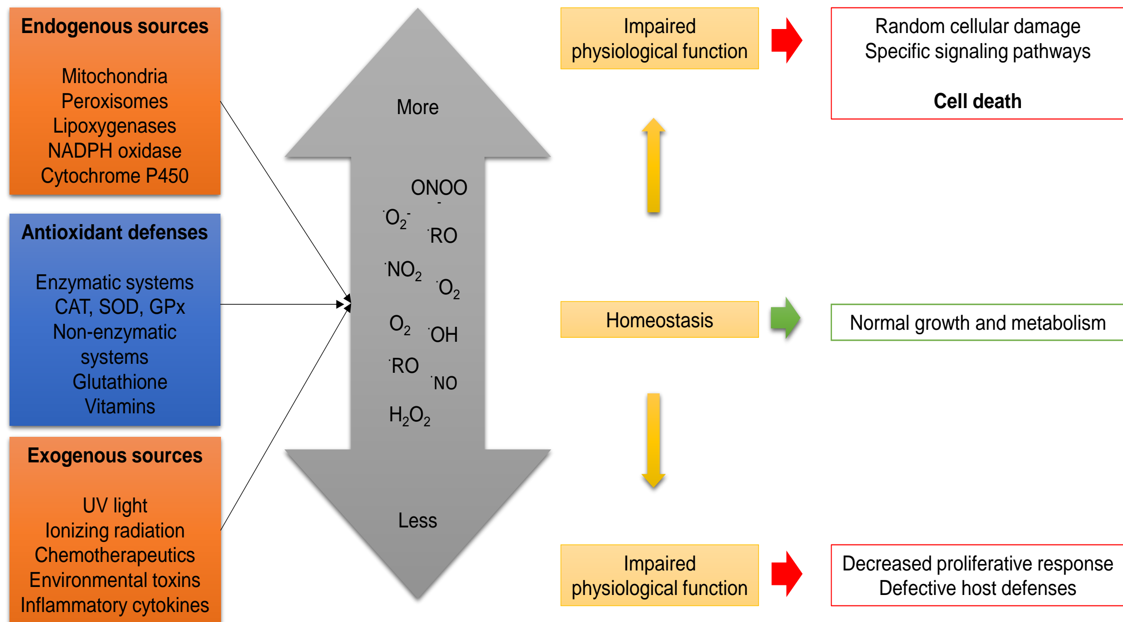


Figure 1. Sources of radical species and possible cellular responses. The oxidative imbalance results from the simultaneous presence of endogenous or exogenous ROS and enzymatic antioxidant defenses like catalase (CAT), superoxide dismutase (SOD) glutathione peroxidase (GPx) or non-enzymatic like glutathione. Diverse defensive mechanisms can be triggered leading to a proliferative decrease up to cell death (adapted from (10)).

While some of the pathways enhance cell survival, others are associated with cell death and many of them can act both ways depending on the circumstances (Table 1). However, it is important to understand that the ultimate response is a result of an integration of multiple stimuli (11).

Table 1. Examples of ROS activated pathways and their contribution to the cellular outcome. From (11).

Signaling pathways[a]	Cellular outcome[b]	
	Enhanced survival	Cell death
p53	+	+++
NFkB	+	+++
HSF1	+++	-
PI3K/Akt	+++	-
ERK	+++	++
JNK	++	+++
p38	+	+
PLCɣ	+++	-
JAK/STAT	+++	-
c-Abl	+	+++

[a] p53: tumor suppressor protein 53; NFkB: nuclear factor kB; HSF1: heat shock transcription factor 1; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; ERK: extracellular signal-regulated kinases; JNK: c-Jun N-terminal kinases; p38: p38 mitogen-activated protein kinases; PLCɣ: phospholipase Cɣ; JAK: Janus protein kinase; STAT: signal transducers and activators of transcription; c-Abl: c-Abl tyrosine kinase. [b] (-): minimal or no evidence of influence; (+): some evidence for the outcome; (++): much evidence that the pathway promotes this outcome; (+++): predominant outcome for this pathway.

Continuous work to discover oxidative stress biomarkers has been developed in order to understand and establish correlations between the mechanisms behind this process, and the possible prevention of its effects (12).

2.1. Molecular damage

2.1.1. Lipid peroxidation

Briefly, lipid peroxidation is a self-propagation chain reaction, representing one of the most harmful toxic phenomena to cellular viability. The polyunsaturated fatty acids (PUFA) located at the cell's membrane are highly affected by this ROS-mediated process. Glycolipids, phospholipids and cholesterol are also well-known targets. The process is initiated when a hydrogen atom is abstracted from a methylene group, resulting on the formation of a carbon radical, which then suffers a rearrangement into a conjugated diene, capable of reacting with oxygen and originating a lipid peroxyl radical ($ROO^{\cdot}$). The chain reaction is propagated when these radicals further abstract hydrogen atoms from other lipids, to form lipid hydroperoxides (ROOH). Also peroxyl radicals can undergo a cyclisation reaction forming endoperoxides, precursors of malondialdehyde, an important marker of oxidative stress able to impair several physiological mechanisms and to react with DNA and proteins (13). Another toxic secondary lipid peroxidation product is 4-hydroxy-2-nonenal (14).

Lipid peroxidation-derived aldehydes can easily diffuse across membranes and covalent bind to proteins in cytoplasm and nucleus. The continuity of this process leads to the loss of membranes' integrity and to possible severe cellular and tecidual injuries (14).

2.1.2. Proteins oxidation

Proteins are also a main target of oxidative stress and due to their ubiquitous location in cells the result of this process can be highly toxic. Besides ROS/RNS, they can also be damaged by sugars and aldehydes. Diverse intracellular pathways may be affected, since the modification of proteins can directly alter the expression of genes *via* modulation of transcription factors. Intrinsically related to proteins oxidation are the mechanisms of protein repair and elimination, with important roles on the pathophysiology

of cardiovascular and immune system diseases, cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's (15).

The modifications suffered by proteins can be generally summarized in three categories (16): i) oxidation of the protein backbone, resulting in protein fragmentation and/or intra or inter-molecular cross-links (Table 2); ii) oxidation of the amino acid side chains, including cysteine and methionine residues (reversible modifications), aromatic amino acid residues and peroxynitrite reactions with methionine, cysteine, tyrosine and tryptophan residues; iii) generation of protein carbonyl derivatives that can result from the oxidative cleavage by either α-amidation or oxidation of glutamyl side chains, directly from the oxidation of sensitive amino acid side chains, by the reaction with aldehydes produced by lipid peroxidation, like 4-hydroxy-2-nonenal and malondialdehyde, and protein glycation (advanced glycation end products) (17).

Again, the final outcome depends on the amount and kind of the oxidants and on the number of alterations induced (18). The result can simply be not significant to alter the native form of the protein or lead to an unfolded protein structure, associated with the loss of functionality and activity (Table 2).

Table 2. Protein damage according to the modifications induced by oxidative stress (adapted from (18)).

Modifications	Properties
Native, unmodified	Active
	Correctly folded form
Oxidized, but bot cross-linked	Progressive oxidative modification
	Increased unfolding
	Increasing loss of activity
Heavily oxidized and cross-linked	Insoluble and undegradable material
	Mainly located in the lysossomal system
	Inhibitory effects on the proteasome
	Showing cytotoxic effects

2.1.3. DNA damage

The permanent damage of the genetic material is known to trigger mutagenesis and carcinogenesis, being implicated several diseases (19).

DNA damage by ROS/RNS is caused by direct interaction with the radical species, either by addition to bases or abstractions of hydrogen atoms from the sugar moieties (20). In DNA bases' damage the ·OH radical is added to the double bond of pyrimidines and purines, especially in high electron density areas, due to its electrophilic nature, resulting in the formation of adducts (Fig. 2). They can also propagate the oxidative process with formation of a large variety of by-products.

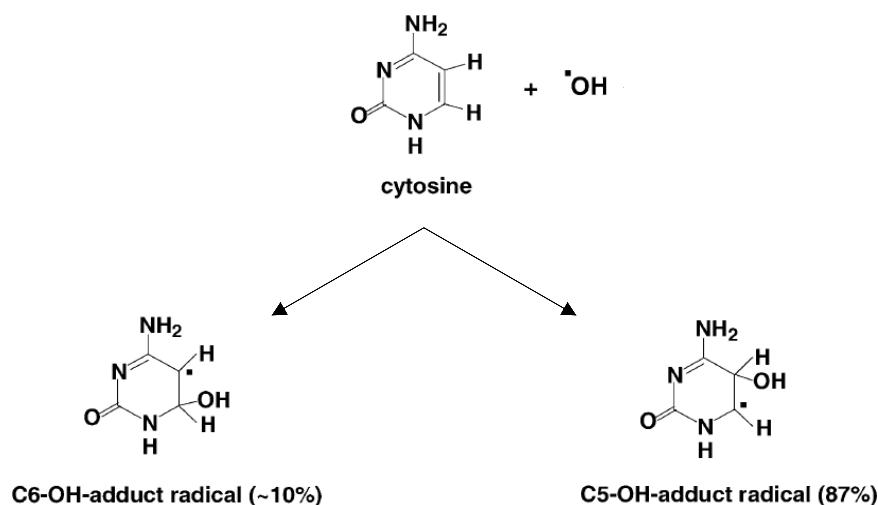


Figure 2. Reaction between the hydroxyl radical and the pyrimidine base cytosine (adapted from (20)).

As for the sugar moieties damage, a hydrogen atom is abstracted from the carbon atoms with formation of carbon-centered radicals, leading to further reactions. As result, the release of free modified sugars can be observed and when not released they can be present as end groups of broken DNA strands, both being on the basis of the DNA strand breakage phenomenon. Moreover, unaltered sugar moieties, base-free sites, can be formed by the elimination of modified DNA bases because of the weakness of the glycosidic bonds. The addition of a DNA base's radical to an aromatic amino acid of proteins or its combination with an amino acid radical, seem to be the mechanisms behind covalent DNA-protein cross-links (20).

One of the most studied biomarkers for DNA oxidative damage is the guanine residue-induced lesion by hydroxyl radical, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), which is pro-mutagenic and relatively easy to form (Fig. 3) (21).

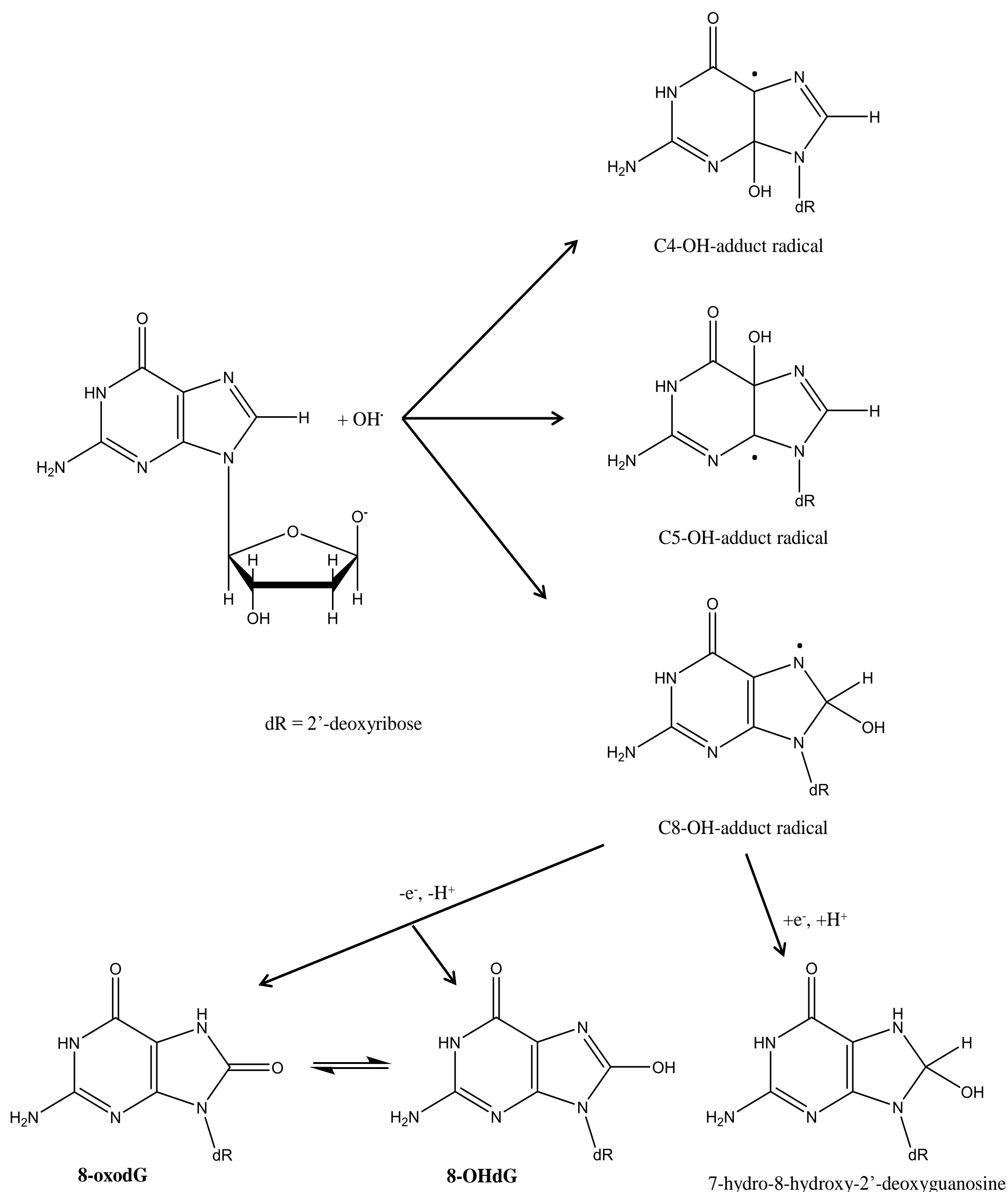


Figure 3. Reactions leading to the formation of the DNA stress biomarkers 8-oxodG and its tautomer 7-hydro-8-hydroxy-2'-deoxyguanosine (8-OHdG). 2'-Deoxyguanosine reacts with hydroxyl radical resulting in the formation of three possible adducts. C8-OH-adduct radical suffers reduction into 7-hydro-8-hydroxy-2'-deoxyguanosine, or into 8-oxodG (favored by keto-enolic tautomerism) and 8-OHdG by oxidation.

There has been a huge development in discovering new molecules and proper techniques to correctly identify and quantify them (22).

2.1.4. Mitochondrial oxidative damage

Mitochondria represent one of the most important sources of ROS and, at the same time, can suffer its damage (23). In the processes to produce ATP through oxidative phosphorylation, electrons are passed in the inner mitochondrial membrane along a series of complexes, known as electron transport chain, to end up reducing oxygen into water (Fig. 4). During this process, single electrons escape from redox centers of the transport chain, $O_2^{\cdot-}$ being formed by a single-electron reduction of molecular oxygen. Among the several complexes, complex I seems to be the main responsible for this phenomenon, being stimulated by succinate, a substrate of complex II, suggesting that an inverse electron flow is involved (Fig. 4).

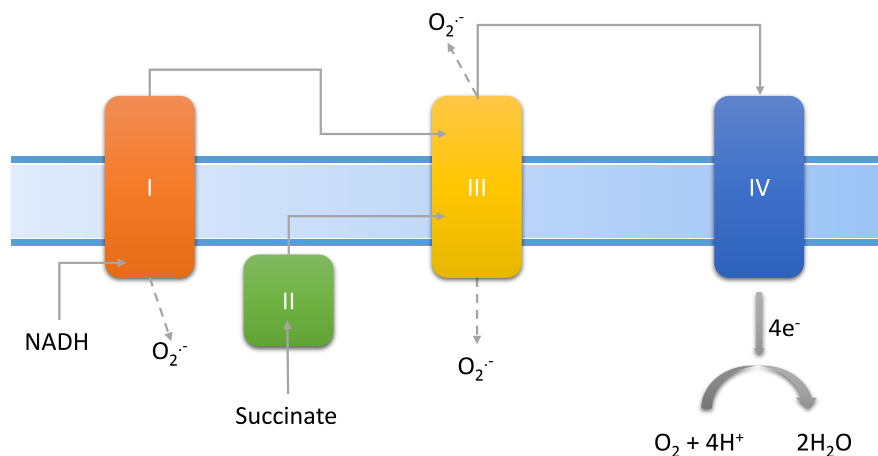


Figure 4. Mitochondrial ROS formation. Complex I is stimulated by succinate, a substrate of Complex II, suggesting that an inverse electron flow is involved (adapted from (23)).

Other ROS species like H_2O_2 can then be originated spontaneously from superoxide radical, or catalyzed by superoxide dismutase (SOD) (24). Moreover, in the presence of ferrous iron (Fe^{2+}), H_2O_2 can precede the formation of hydroxyl radicals *via* Fenton reaction. Additionally, monoamine oxidase, present in the outer mitochondrial membrane, is an important source of ROS, by oxidative deamination of primary aromatic amines, tertiary cyclic amines and long-chain diamines.

Once mitochondrial antioxidant systems are overwhelmed by ROS, the oxidative damage can occur. Besides de molecular damages described before, mitochondria are affected in some specific targets. Mitochondrial DNA is extremely sensitive, since it is proximate to the electron transport chain and lacks protection from histones. Its damage can affect cellular vital processes like ATP production and cause disturbance of the mitochondrial membrane potential. As examples of other complex toxic mechanisms

triggered by ROS exposure are the inactivation of aconitases (iron-sulfur proteins), with the consequent release of iron, and the loss of calcium homeostasis (23).

2.2. Antioxidant mechanisms

As discussed above, cells have developed defensive mechanisms against oxidative stress. Attending to their origin they can be classified as endogenous or exogenous and can actuate either in a preventive manner, as radical scavengers or even in reparation processes (25). Preventive antioxidants reduce de radical species formation, act by metal ions chelation and can mediate in dismutation reactions. As for radical scavengers, they play a pivotal role stopping the lipid peroxidation chain reactions due to the formation of more chemical stable molecules. The last group can repair oxidative damage induced by ROS on proteins, DNA and cell's membrane.

One of the must studied non-enzymatic endogenous antioxidants is glutathione (Fig. 5), ubiquitously present in the nucleus, endoplasmic reticulum and mitochondria of all cells. It is physiologically important for the maintenance of the essential thiol status of proteins, detoxification mechanisms, provides a reservoir for cysteine, modulates the activity of neurotransmitter receptors and is involved in diverse cellular processes, like DNA synthesis and immune functions (26). Its synthesis occurs by two sequential ATP-dependent reactions catalyzed by ɣ-glutamylcysteine synthetase (rate-limiting enzyme) and glutathione synthetase (26).

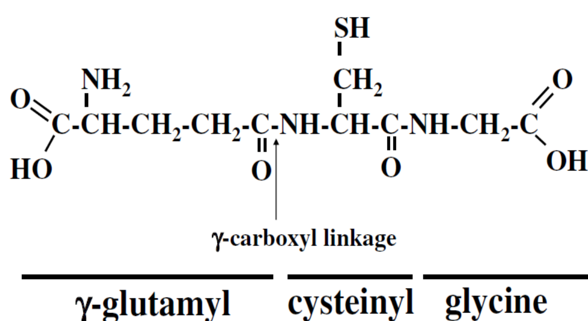


Figure 5. Structure of the tripeptide ɣ-L-glutamyl-L-cysteinyl-glycine or glutathione.

As antioxidant, it is a primary line of defense and is found at a reduced state (GSH) and oxidized state (GSSG), also associated with proteins acting as coenzyme for the denominated glutathione-dependent enzymes (Fig. 6).

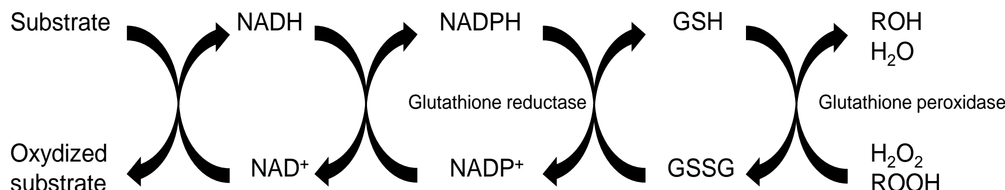


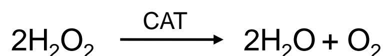
Figure 6. Glutathione redox-cycle and antioxidant activity.

Ascorbic acid, α-tocopherol and β-carotene are also examples of other non-enzymatic antioxidants (27).

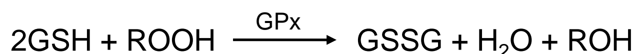
As for enzymatic endogenous mechanisms, SOD, catalase (CAT) and GSH-dependent enzymes are considered the most important antioxidants. Their main purpose is to act against ROS.

Superoxide dismutase enzymes are classified according to their constitutive metal ion: Cu/Zn-SOD, Mn-SOD and Fe-SOD. They catalyze superoxide radical dismutase into hydrogen peroxide and oxygen:

Catalase enzyme can either catalyze hydrogen peroxide or oxidize hydrogen donors with hydrogen peroxide consumption:



Glutathione peroxidases (GPx) are a family of selenoproteins able to reduce hydroperoxides to the corresponding hydroxyl compounds:



They have particular notoriety in the gastrointestinal tract as defense against diet-derived hydroperoxides or xenobiotic metabolites. During this process selenium is oxidized and a seleno-dissulfide is formed, which is then cleaved by a second GSH molecule yielding the reduced GPx (28).

Glutathione S-transferases (GST) are known by their ability to detoxify xenobiotics and to inactivate endogenous reactive molecules. They constitute a second line of defense against oxidative stress since they catalyze the conjugation of GSH with oxidation end-products (28).

Despite the *de novo* synthesis, GSH levels can be maintained through a reaction catalyzed by glutathione reductase (GR):

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The role of phenolic compounds, as representatives of exogenous antioxidants, will be discussed forward.

2.3. Antioxidant activity assessment

Distinct methods and experimental models have been developed in order to assess the antioxidant activity of a given compound/extract (29). The more classic ones consisted on the measurement and presentation of the results as total antioxidant capacity or total antioxidant activity. The reductive and radical scavenging activities can be classified in three main groups: i) ability to reduce metal ions, including the ferric ion reducing antioxidant power (FRAP), ii) ability to reduce organic radicals, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\cdot+}$) radicals being the most commonly used, and iii) peroxyl radical reduction ability assays, like the oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP).

Other parameters, such as metal ions chelating activity, inhibition of free radical generating enzymes and activation of internal antioxidant enzymes like CAT, SOD and GSH-dependent enzymes are also frequently applied.

Nowadays, determinations based only on a chemical approach are used as screening tools for cell based systems, since they do not take into consideration bioavailability and metabolic variables (30).

3. Phenolic Compounds

Concerning to the most studied chemical families of natural bioactive compounds, polyphenols are certainly the best example. With ubiquitous distribution and comprising molecules from low molecular weight to more than 30000 Da, they are particularly known for their antioxidant (31), anti-inflammatory (32) and antimicrobial (33) properties. These secondary metabolites are biosynthesized *via* shikimate and acetate pathways. They can present simple structures like the phenolic acids, or complex polymeric ones characteristic of tannins. Additionally, in heterosides' structure the aglycone can be linked to sugar residues either by their hydroxyl groups or by carbon atoms of the main skeleton. Although glucose is the most common sugar, others like rhamnose, galactose, xylose and glucuronic acid are frequently observed (34).

3.1. Flavonoids and hydroxycinnamic acids

Flavonoids are phenolic compounds with a common structure known as 2-phenil-benzopirane (C_6-C_3-C_6), consisting in two aromatic rings linked by three carbons, usually forming an oxygenated heterocycle (Fig. 7) (34). The A ring usually comes from a resorcinol or phloroglucinol *via* acetate pathway, while the B ring is biosynthesized by the shikimate pathway (34). These compounds can be sub-classified according to different substitutions in the main structure (Fig. 7).

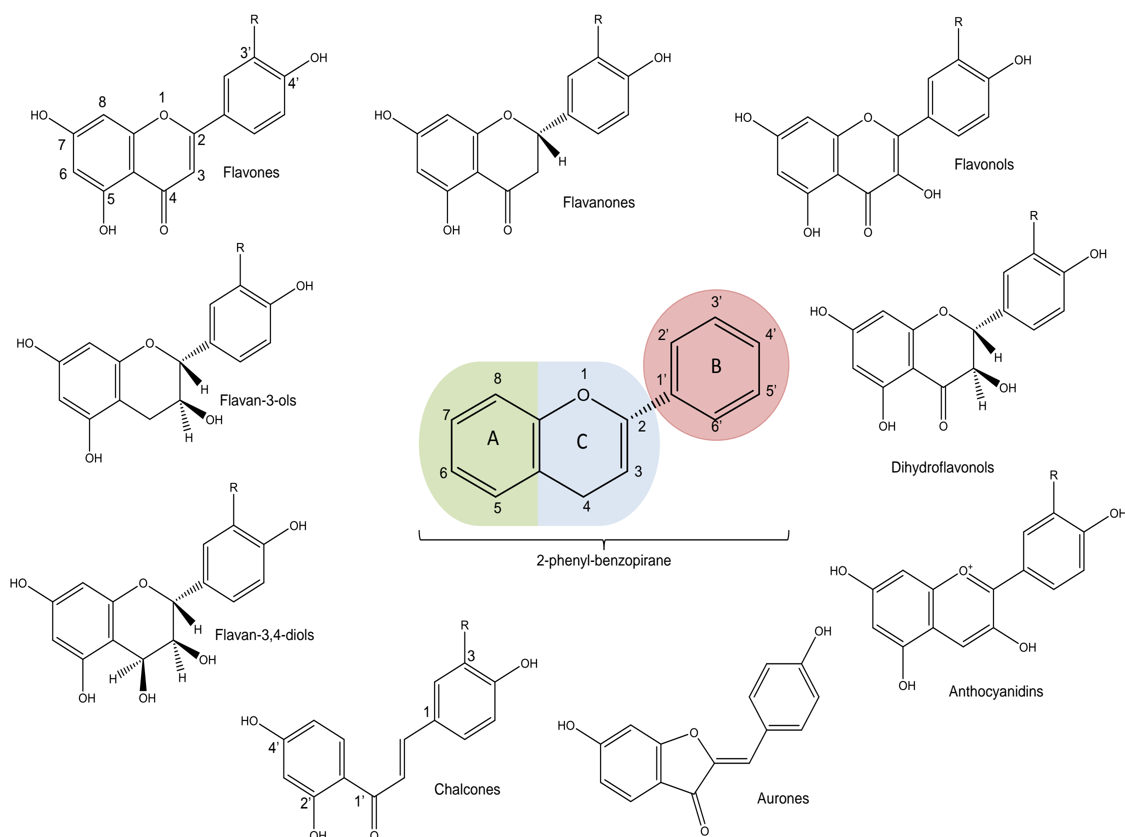


Figure 7. Common structure of flavonoids (2-phenyl-benzopirane), and main sub-classes.

Among the phelylpropanoid derivatives (C_6-C_3) the hydroxycinnamic acids are considered to be the most important compounds (Fig. 8) (35).

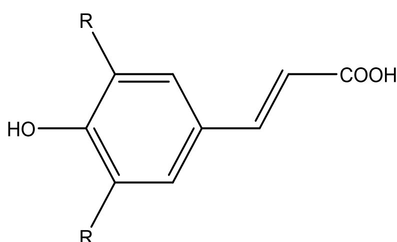


Figure 8. Basic structure of hydroxycinnamic derivatives.

3.2. Phenolic compounds as antioxidants

Behind the antioxidant properties of phenolics underlies a conjugation of a vast number of mechanisms. Their characteristic hydroxyl groups easily donate hydrogen atoms to radical species, stopping the chain reactions due to the formation of a non-

propagating radical. Another mechanism is by metal ions' chelation, despite that sometimes it leads to pro-oxidant activity (36).

Recent studies show that besides this more classic mechanisms, phenolic compounds can interact with biological systems by modulation of signal transduction pathways, or even interaction with cell receptors. Moreover, phenolic compounds seem to be able of endogenous cell antioxidants induction, like sulfotransferases, epoxide hydrolases and GSH and GSH-related enzymes, through the modulation of antioxidant responsive elements located in the promoters of diverse genes (28).

Focusing on flavonoids, their antioxidant activity is intrinsically correlated to their chemical structure. Generally, aglycones are more potent antioxidants than their corresponding glycosides. The number and configuration of hydroxyl groups influences the antioxidant activity, B-ring configuration as 3',4'-cathecol structure seeming to be the most determinant (Fig. 9) (37).

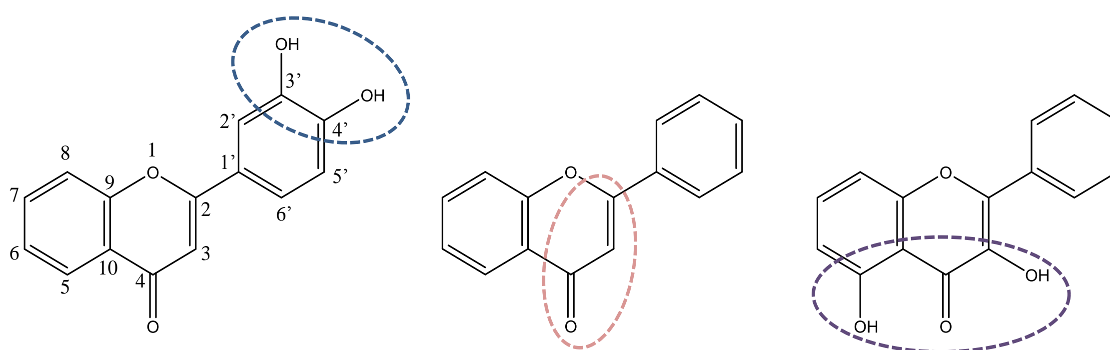


Figure 9. Structure-activity relationship of flavonoids.

A greater stability of the flavonoid radical is observed when there are present a carbonyl group at position 4 and a 2-3 double bound between A and B rings, due to the resonance effect of the aromatic nucleus (Fig. 9) (37). Furthermore, differences in the hydrophobicity and molecular planarity due to substitutions either by hydroxyl or methoxyl groups have been noticed. When a methoxyl group is present at the B-ring, the scavenging activity is diminished by the steric obstruction caused to the 3',4'-cathecol structure, when compared to the ones with hydroxyl substitutions (37).

Some of the same features can be applied to hydroxycinnamic derivatives. The highest antioxidant activity is related with the presence of more hydroxyl groups, especially on *ortho* positions, but contrary to flavonoids it is enhanced by methoxyl substitutions on the catechol group (38). The propenoic side chain in hydroxycinnamic

derivatives, instead of the carboxylic group of benzoic acid derivatives, provides additional stability by resonance on the phenoxyl radical, thus highest activity (38).

4. The genus *Mentha*

Mentha genus is one of the most important and studied within Lamiaceae (Labiatae) family, in the Order Lamiales. Its species are commonly known as mints due to their characteristic menthol flavor (39). Mints have an important commercial value, since they are used in industry, medicine and agriculture. In the Northwest of the Iberian Peninsula they have a particular application in gastronomy as aromatic herbs, being also consumed as herbal teas (40, 41).

The systematics of this genus is not consensual. Regarding the number of species included, it varies depending on the type of study, being rather morphological, cytological, anatomical, phytochemical or genetic (42). The complex classification is based on the ease of hybridization favored by gynodioecy, that despite the majority of infertile specimens created, has a strong capacity of dissemination by vegetative propagation in Nature and crop species (43). The last proposed classification refers to the presence of 18 species and 11 hybrids, distributed by four sections: *Mentha*, *Eriodontes*, *Tubulosae* and *Pulegium* (44).

Concerning mints as sources of bioactive compounds, they provide a distinct number of molecules like alkaloids, flavonoids, phenolic acids and terpenoids (45). The most part of the scientific studies approaching the pharmacological and toxicological potential of these species focus on their essential oils. Different activities, such as insecticidal (46), antimicrobial (47), anti-inflammatory (48), analgesic (49), and antioxidant (50), are well established. Also, essential oils are highly toxic and the ingestion of large amounts results on acute toxicity with allergic reactions and hepatic injuries (51).

One traditional way for mints classification is based on the analysis of the chemical composition of the essential oils, identifying which is the major monoterpenoid present and respective metabolic pathway. There by, the species can be categorized in three groups: one with major expression of the menthol pathway, other with major expression of the carbon pathway, and the last with major expressing of the linalool pathway (Table 3).

Table 3. Mints classification according to the major monoterpenoid present in the essential oil and its metabolic pathway (adapted from (42)).

Menthol pathway	Carvon pathway	Linalool pathway
Mentha aquatica L.	*Mentha spicata* L.	*Mentha* x *piperita* (L.) Huds var. *lavanduliora* ined.
Mentha arvenses L.	*Mentha* x *villosa* Huds.	
Mentha longifólia L.	*Mentha suaveolens* Rhr.	*Mentha* x *aquatica* var. *citrata* (Ehrh.) Frensen.
Mentha pulegium L.		
Mentha x *piperita* L.		
Mentha x *verticillata* L.		

Mentha pulegium L. has particular interest in the Mediterranean and Portuguese cultures (Fig. 10). It is an herbaceous species with height ranging between 20 and 40 cm, strongly aromatic and little elliptic oblongated leaves (8 to 30 mm length); it has characteristic spherical verticillated inflorescences, ciliate calyx-teeth and a lilac corolla measuring around 5 cm; it can be easily found in Europe and Western Asia, with large distribution in continental Portugal and Azores; its habitat consists in high humid areas, mostly near water courses (52).

Figure 10. *Mentha pulegium* Linnaeus.

In phytotherapy, the flowered aerial parts and the essential oil are used to treat and prevent diverse gastrointestinal disorders like the lack of appetite, intestinal cramps, low intestinal motility, flatulence and dyspepsia, and to alleviate cold and flu-related symptoms. External application is due to their antiseptic and healing properties. In Portugal, popularly known as "poejo", it is used in gastronomy as an aromatic herb in the regions of Trás-os-Montes and Alentejo, consumed as an alcoholic beverage ("licôr de

poejo”) and herbal tea, being also used as parasites and insects’ repellent at livestock farms (53).

M. pulegium essential oil, or pennyroyal oil, has emmenagogue and abortifacient properties. It is used by women to induce menstruation and abortion (54). The ingestion of high doses can lead to acute intoxication and death, owing to the presence of pulegone and its bioactive metabolite menthofuran, both strong hepatotoxic agents (55).

4.1. *Mentha pulegium* L. chemical profile

4.1.1. Terpenoids

The vast class of terpenoids includes more than 40.000 different known structures. Synthesized by plants, they have a huge impact in their life cycle as primary and secondary metabolites. Among other biological functions, they play important roles as membrane’s constituents, hormones, photosynthetic pigments, in communication and as natural mechanisms of defense. Widely found in medicinal plants’ essential oils, they are of great importance to the pharmaceutical and food processing industries (56).

Terpenoids are biosynthesized by mevalonate (cytosolic) and non-mevalonate (plastidic) pathways, both converging on the isoprene formation, the basic functional unit. The addition of isopentenyl pyrophosphate units to its isomer dimethylallyl pyrophosphate results in new precursors of other complex terpenoids, *via* prenyltranferases. Based on the number of isoprene units (5 carbons) present on the structure, terpenoids can be sub-classified into several groups (Fig. 11).

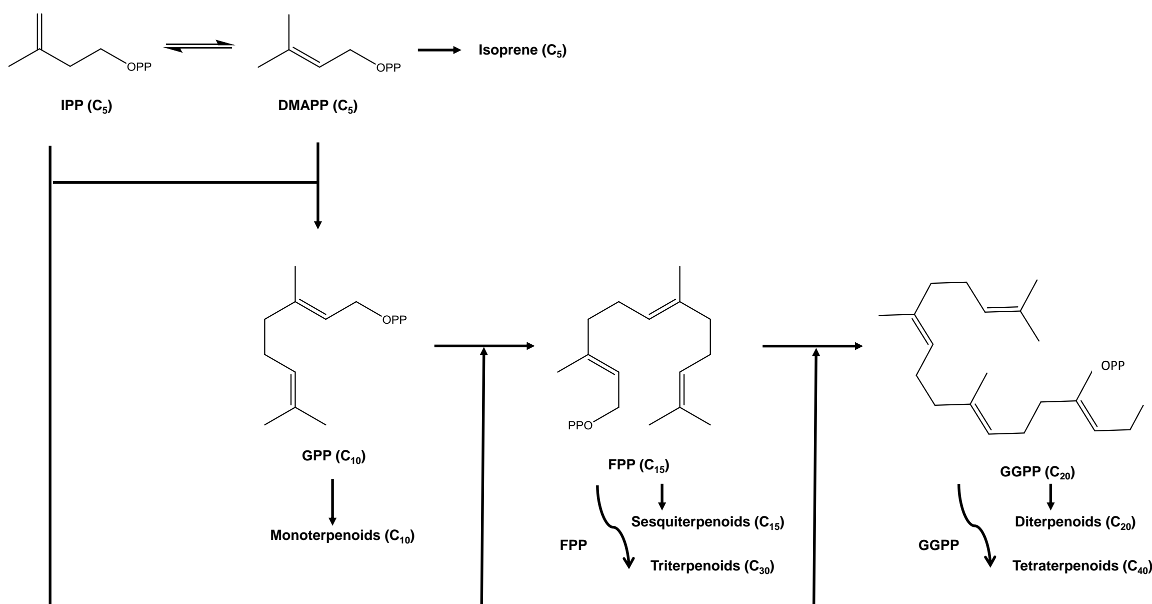


Figure 11. Terpenoids biosynthesis. From the condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), higher terpenoids precursors are formed. Geranyl pyrophosphate (GPP) is the monoterpenoids' precursor, farnesyl pyrophosphate (FPP) is the sesquiterpenoids' precursor and geranylgeranyl pyrophosphate (GGPP) is the diterpenoids' precursor. Squalene, being the precursor of triterpenoids, results from de condensation of two FPP units. Two GGPP units condense to form tetraterpenoids (adapted from (56)).

As previously described, mints can also be classified by the major monoterpenoids present in their essential oils. In the essential oil of *M. pulegium* pulegone, menthone, piperitenone and piperitone (Fig. 12) are the main monoterpenoids, biosynthesized by the menthol pathway. According to the oxygenated monoterpenoid present, this species can also be classified in 3 distinct chemotypes: pulegone type, piperitone/piperitenone type and isomenthone/neo-isomenthol type (44).

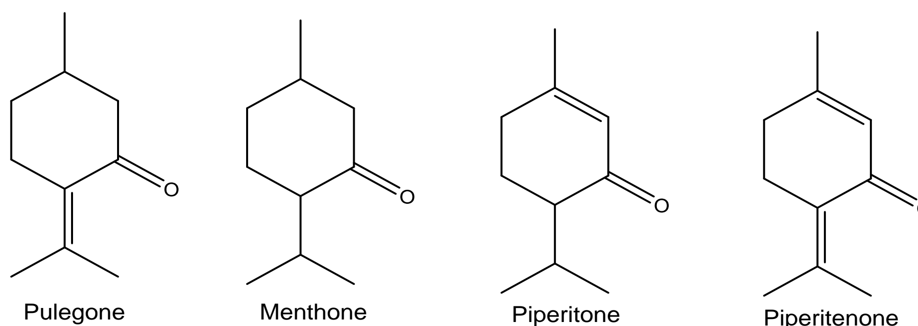


Figure 12. Major monoterpenoids present in the essential oil of *M. pulegium*.

Other classes, such as hydrocarbonated monoterpenes (limonene, α-pinene and camphene), sesquiterpenes (β-borbene), oxygenated sesquiterpenes (palustrol and α-cadinol), oxygenated diterpenes (epimanoyl oxide), among others, can also be found in

the aerial parts of the plant (57). Recently, new terpenoids have been identified in the leaves, but no significant antioxidant or antimicrobial activity was described (58). As essential oils are obtained from natural sources, their chemical composition can vary according to the physical-chemistry characteristics of soil, climate and maturation stage of the species (59).

4.1.2. Phenolic compounds

As for the phenolic profile of *M. pulegium*, a lot of work has to be done. A few number of compounds has been identified and the literature on this subject is scarce and lacks consistency. Most of the studies available are directed to the total quantification of phenolic compounds and not to their structural characterization, leaves being the most studied material. The presence of flavonoids and phenolic acids has already been reported for this species' extracts.

Phenolic acids, such as caffeic, vanillic, ferulic, rosmarinic and lithospermic, and the flavonoids luteolin, apigenin, naringenin, (+)-catechin and diosmetin 7-*O*-rutinoside are some examples of the compounds already identified (Fig. 13) (60-65). Also jaceosidin, pectolinarigenin and pedalitin were found in *M. pulegium* leaves' alcoholic extracts (66).

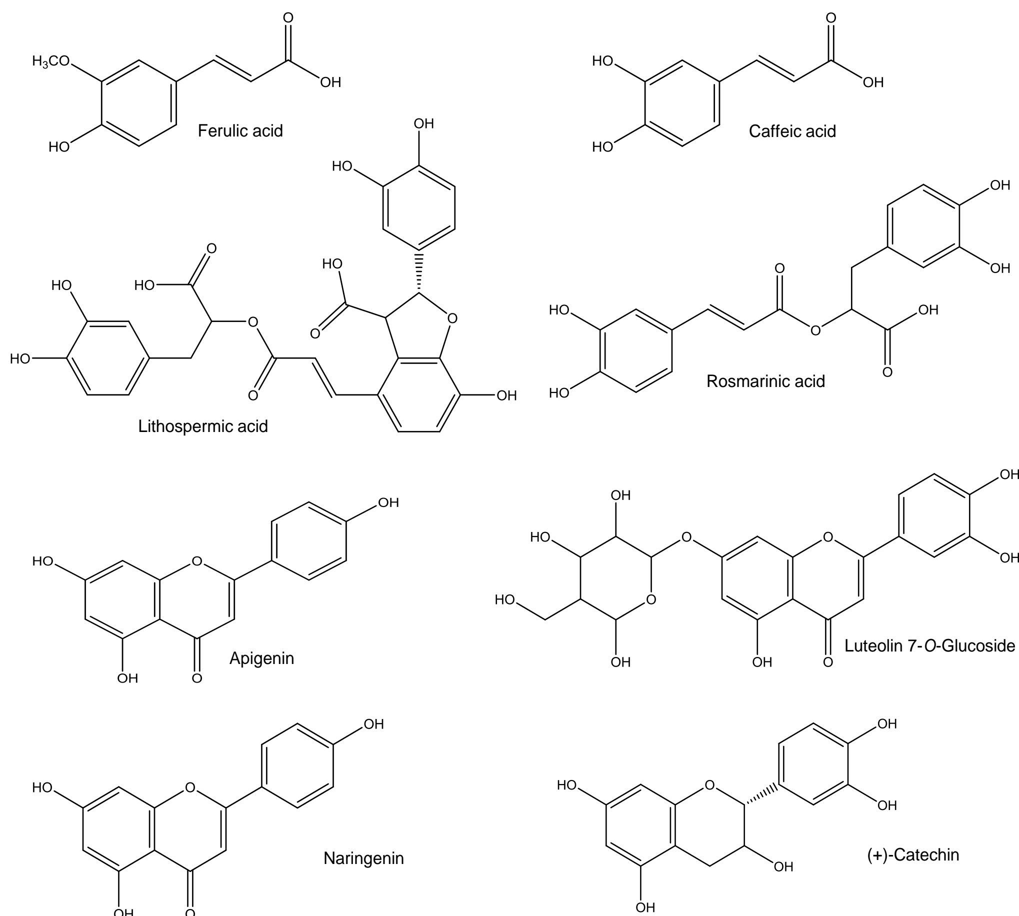


Figure 13. Chemical structures of some phenolic compounds identified in *M. pulegium* extracts.

4.2. Pharmacologic and toxic activities

4.2.1. Antioxidant activity

As above mentioned, there are available a variety of *in vitro* assays to assess the antioxidant activity of extracts, sometimes making hard to correlate the results obtained. Here will be presented some examples of works concerning diverse extracts of *M. pulegium*.

The potential to scavenge DPPH· radical was evaluated for the methanolic extracts of flowered aerial parts of mint species (67). When compared to the other mints, *M. pulegium* showed a lower ability; nevertheless, 76% of scavenging activity was found at 0.4 mg/mL, a very promising result. Neither the chemical profile of each extract nor the phenolic quantification were determined, rendering impossible an attempt to link the activities with the compounds present.

In another work, with the methanolic extract of *M. pulegium* at flowering stage, it was confirmed a significant scavenging ability against DPPH$^{\cdot}$ and ABTS$^{\cdot +}$ radicals and a marked capacity to chelate metal ions (68). The hexane extract demonstrated a poor potential in all *in vitro* assays and the lowest phenolic content.

The antioxidant potential of the essential oil, assessed by DPPH$^{\cdot}$ assay, was lower than that of the methanolic extract (69).

In a more complete analysis, the temperature of extraction seemed to be an important variable to obtain better antiradical activities (57). Polar extracts were richer in phenolic compounds than the essential oil and generally more active. Additionally, the hot water extract presented higher phenolic content and more marked activities than the cold water extract (57).

Traditionally herbal teas are prepared with hot water. Hence an efficient extraction of polyphenols can be achieved, with great benefits for consumers.

The antioxidant activity was also assayed *in vivo* (70). Albino Wistar rats were treated for seven days by oral administration of an ethanolic extract from *M. pulegium* leaves. The toxicity was than induced with CCl_4. At 600 mg/Kg the extract was able to protect against the toxicant. The monitored biomarkers, glutathione, SOD, CAT and peroxidase, were proximal to control's basal values, and the ethanolic extract was not toxic up to 2000 mg/Kg. However, the authors did not proceed with a histologic analyses that could sustain a relation between blood concentrations and actual tecidual damage.

4.2.2. Antigenotoxic and antimutagenic activities

The somatic mutation and recombination test (SMART) was applied to evaluate the genotoxicity of *M. pulegium* aqueous extract (71). The results showed that it was not genotoxic: in fact, it was demutagenic when assayed in co-exposition with hydrogen peroxide, thus preventing oxidative stress.

The protective effect against CCl_4-induced toxicity was also studied in human peripheral blood lymphocytes (72). Through the sister chromatid exchange (SCE) test, the methanolic extract demonstrated a very significant anti-genotoxic activity, reversing the CCl_4 induced damage. Additionally, the activities of antioxidant related enzymes (SOD and GPx) and GSH levels were evaluated and the oxidative stress biomarker malondialdehyde was quantified. As expected, when cells were treated with CCl_4 alone the enzymatic activity decreased and the levels of malondialdehyde significantly

increased. On the other hand, when co-exposed to both methanolic extract and CCl_4, an increase of enzymatic activities and a decrease of malondialdehyde levels was observed, suggesting an antioxidant protective role mediated by the extract.

In another work the antimutagenic activity of methanolic and hexane extracts was evaluated against Fenton reagent (68). The DNA nicking assay technique was applied, and it was observed a significant reduction in the formation of plasmid pBR 322 nicked DNA, and increased native form. Furthermore the observed protective effect was more prominent for the methanolic extract than for the hexane extract. Phenolic compounds share more polarity similarities with methanol, and effectively the methanolic extract presented a higher phenolic content and more effective radical scavenging and metal chelation activities. It was suggested that these mechanisms were behind the protection against the lethal effects of oxidative stress by the generated hydroxyl radical.

4.2.3. Cytotoxicity

Diverse cell lines have already been subjected to *M. pulegium* extracts exposure. According to the results obtained in the MTT viability assay, the essential oil was more toxic for the cell lines Vero (green African monkey kidney), HeLa (human malignant cervix carcinoma) and HEp-2 (human laryngeal carcinoma) than the methanolic extract (73). In this study the lowest most sensitive cells were those from Vero (extract LC_{50} = 51.2 µg/mL) and HeLa (essential oil LC_{50} = 30.6 µg/mL) lines.

In another study the cell lines HeLa, SK-OV-3 (human ovarian adenocarcinoma) and A549 (human lung carcinoma) were also exposed to both methanolic extract and essential oil of flowered parts (74). By using different viability assays, clonogenic and neutral red vital dye, no cytotoxic effects were observed when exposed to the extract. SK-OV-3 and A549 cells were the most sensitive to essential oil exposure, LC_{50} values of 14.4 and 18.76 µg/mL being found, respectively.

The methanolic extract also did not lead to significant viability decrease of Caco-2 (human colon carcinoma), HepG2 (human hepatoblastoma) and MCF7 (human breast cancer) cells, as observed with crystal violet dye (75).

4.2.4. Antimicrobial and insecticide activities

As discussed before, *M. pulegium* is also traditionally used as an insect repellent at live stocks farms. The essential oil is particularly active against some species, such as *Mayetiola destructor*, *Dermatophgoides farina*, *Malus domestica* and *Drosophila melanogaster* (76). Other extracts, like hexane and methanolic, are also active but less effective, which is explained by the lipophilic nature of the essential oil and its strong impairment to insects' metabolism and physiologic and behavior regulation. Moreover oxygenated monoterpenoids, like pulegone (Fig. 14), are more active than the non-oxygenated ones. Monoterpenoids are toxic to insects central nervous system and metabolized by cytochrome p450, being also strong inhibitors of acetylcholinesterase (AChE) and octopamine receptors (77).

The increasing microbial resistance to antibiotics makes necessary the pursuit of new active molecules. In a study focusing *M. pulegium* essential oil chemical characterization, as well as its antibacterial potential, it was reported, as expected, that Gram-negative bacteria were less sensitive than Gram-positive bacteria, with the exceptions of *Vibrio cholera* and *Listeria monocytogenes* (78). The best activities were obtained against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The results were attributed mainly to the presence of piperitone and piperitenone (Fig. 14).

Different strains were also tested to compare the antibacterial potential between the essential oil and different extracts. From the results obtained it could be observed that all microorganisms presented extraordinary more sensibility to the essential oil than to aqueous and ethanolic extracts exposure. Behind such activity was the presence of menthone, pulegone and neomenthol, compounds able to cause cellular membrane damages (57).

The essential oil obtained from *M. pulegium* leaves was very effective against *Malassezia furfur* fungus, with a 0.5 cm inhibition halo radius at 2 mg/mL (79).

4.2.5. Other activities

The ability to inhibit AChE was determined for *M. pulegium* essential oil, ethanolic and aqueous extracts, along with other four aromatic herbs (80). For all the species, the best activities were obtained with the essential oils, followed by ethanol extracts and

aqueous extracts. Specifically for *M. pulegium* the IC_{50} values were 324 µg/mL, 534 µg/mL and 1581 µg/mL, respectively, thus showing a moderate anticholinesterase activity.

Aiming to investigate potential central nervous system activities, the protective effect of the methanolic extract was tested against hydrogen peroxide-induced toxicity in PC12 cells (81). The capacity to inhibit monoamine oxidase A (MAO-A), AChE and the binding affinity to ɣ-aminobutyric acid A receptor ($GABA_A$) were also evaluated. When co-exposed to H_2O_2 and methanolic extract (1000 µg/mL) a statistically significant protection effect was observed. As for MAO-A inhibition, a dose-dependent activity was noticed (IC_{50}= 59 µg/mL), but no relevant results were obtained for the ability to bind to $GABA_A$. Unlike the results obtained with the previous, the methanolic extract did not inhibit AChE. Among the five mints analyzed *M. pulegium* showed some of the less relevant neuroprotective and neurochemical activities.

Phenolic compounds are known for their strong metal ion chelating property. When in the intestinal lumen, they can easily complex with iron, resulting in lower absorption. A study was developed to measure the effects on iron absorption resulting from the ingestion of polyphenol-rich beverages, based on an extrinsic tag radio-Fe technique (^{55}Fe, ^{59}Fe) to evaluate erythrocyte incorporation (82). *M. pulegium* infusion was able to reduce iron absorption by 73% when compared to water control ingestion, leading the authors to admonish the possible risk of its consumption by anemic patients.

Chapter II - Objectives

The main objectives of this dissertation were:

- ✓ To establish the phenolic profile of *M. pulegium* infusion;
- ✓ To evaluate the response of Caco-2 and AGS cell lines to the infusion exposure;
- ✓ To verify the protective potential against *tert*-butylhydroperoxide-induced toxicity;
- ✓ To understand the mechanisms behind the antioxidant activity;
- ✓ To check the presence of intracellular phenolic compounds;
- ✓ To co-relate the chemical profile with the observed biological activities.

Chapter III - Experimental section

1. Materials and methods

1.1. Standards and reagents

Sodium pyruvate, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), β-nicotinamide adenine dinucleotide reduced form (NADH), *tert*-butylhydroperoxide (*t*-BHP), sodium nitroprusside dehydrate (SNP), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, amphotericin B (250 µg/mL), transferrin (4 mg/mL), non-essential amino acids, methanol, formic acid, sodium hydroxide, acetonitrile, formaldehyde, perchloric acid, dimethyl sulfoxide (DMSO), 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), 2-aminoethyl diphenylborinate (DPBA), GSH, GSSG, glutathione reductase (EC 1.6.4.2), caffeic, chlorogenic and rosmarinic acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ferulic acid, luteolin 7-*O*-glucoside and apigenin 7-*O*-glucoside was from Extrasynthese (Genay, France) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) from Alfa Aesar (Karlsruhe, Germany). Dulbecco’s Modified Eagle Medium (DMEM), Dulbecco’s phosphate buffered saline (DPBS), Hank's Balanced Salt Solution (HBSS), fetal bovine serum (FBS), Pen-Strep solution (Penicillin 5000 units/mL and Streptomycin 5000 mg/mL) and trypsin-EDTA were purchased from Gibco (Invitrogen, Paisley, UK). Water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Human epithelial colorectal adenocarcinoma (Caco-2) and Human epithelial gastric adenocarcinoma (AGS) cell lines were acquired from American Type Culture Collection (ATCC).

1.2. Samples and extract preparation

Specimens were kindly collected and provided by Direção Regional de Agricultura entre Douro e Minho (DRAEM). The samples were dried at 30 ºC for 24 hours followed by powdering, and stored protected from light and humidity. In order to mimic the herbal tea usually prepared for human consumption, 200 mL of boiling water were added to 4.00 g of the powdered material and left to stand for 15 min. After filtration the obtained extract was frozen at -20 ºC and then lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA).

1.3. HPLC-DAD-ESI/MS^n qualitative analysis of phenolic compounds

HPLC-DAD-ESI/MS^n chromatographic analysis was carried out on a Kinetex column (5 µm, C18, 100 Å, 150 x 4.6 mm; Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents: water-formic acid (1%) (A) and acetonitrile-formic acid (1%) (B), starting with 10% B and using a gradient to obtain 30% B at 20 min. The flow rate was 1 mL/min and the injection volume 20 µL. Spectral data from all peaks were accumulated in the range of 240-400 nm and chromatograms were recorded at 330 nm. The analyses were carried out in an Agilent HPLC 1100 series equipped with a diode array detector 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 was an ion trap spectrometer (model G2445A) 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. Mass spectrometry data were acquired in the negative ionization mode. MS^n was carried out in the automatic mode on the more abundant fragment ion in MS$^{(n-1)}$.

1.4. HPLC-DAD quantitative analysis of phenolic compounds

HPLC-DAD conditions were the same as those described above, except for the Waters Spherisorb ODS2 (25.0 x 0.46 cm; 5 µm particle size) column. 20 µL of the lyophilized extract dissolved in water (25 mg/mL) were injected into a HPLC-DAD unit (Gilson), spectral data were acquired with a Gilson-DAD and processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Phenolic compounds quantification was achieved by the absorbance recorded at 320 nm and 350 nm for phenolic acids and flavonoids, respectively, in relation to external calibration curves (Table 4). Since commercial standards were not available for all the compounds, caffeic acid glucosides, trimmers and tetramer were quantified as caffeic acid, ferulic acid glucosides as ferulic acid, luteolin and apigenin derivatives as luteolin 7-*O*-glucoside and apigenin 7-

O-glucoside, respectively. Caffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Rosmarinic acid and its derivatives were quantified as rosmarinic acid.

Table 4. Regression equations, LOD and LOQ for phenolic compounds quantification.

Compounds[a]	Regression equation	R^2	Range of concentrations (mg/mL)	LOD[b] (mg/mL)	LOQ[c] (mg/mL)
5-Caffquin Ac	$7.2 \times 10^8\ x - 3.1 \times 10^6$	0.994	$1.2 \times 10^{-1} – 1.5 \times 10^{-2}$	4.3×10^{-4}	1.3×10^{-3}
Caffeic Ac	$1.5 \times 10^9\ x + 7.5 \times 10^6$	0.992	$1.6 \times 10^{-1} – 8.0 \times 10^{-3}$	6.4×10^{-4}	1.9×10^{-3}
Ferulic Ac	$1.4 \times 10^9\ x + 6.2 \times 10^6$	0.994	$1.3 \times 10^{-1} – 6.0 \times 10^{-3}$	4.7×10^{-4}	1.4×10^{-3}
Rosmarinic Ac	$8.2 \times 10^8\ x + 6.1 \times 10^6$	0.994	$2.1 \times 10^{-1} – 1.1 \times 10^{-2}$	1.3×10^{-3}	1.4×10^{-3}
Lut-7-Gluc	$4.6 \times 10^8\ x + 2.0 \times 10^6$	0.999	$1.20 \times 10^{-1} – 6.0 \times 10^{-3}$	1.1×10^{-4}	3.4×10^{-4}
Apig-7-Gluc	$8.5 \times 10^8\ x - 1.5 \times 10^6$	0.997	$1.4 \times 10^{-1} – 1.7 \times 10^{-2}$	4.3×10^{-4}	1.3×10^{-3}

[a] Caffquin: caffeoylquinic; Ac: acid; Lut: luteolin; Apig: apigenin; Gluc: glucoside.

[b] Limit of detection.

[c] Limit of quantification.

1.5. Antiradical activity

1.5.1. DPPH$^{\bullet}$ scavenging assay

DPPH$^{\cdot}$ is a free radical able to accept an electron or hydrogen atom becoming a non-radical and hardly oxidizable species.

The scavenging activity was determined spectrophotometrically (83). The assay was performed in 96-well plates and a set of extracts dilutions was prepared. The reaction mixture consisted on 25 µL of extract (redissolved in water) and 200 µL of DPPH$^{\cdot}$ methanolic solution (150 mM). The plates were incubated for 30 min with light absence, and the absorbance at 515 nm was determined with a Multiskan Ascent plate reader (Thermo Electron Corporation, Vantaa, Finland). Three experiments were performed in triplicate.

1.5.2. Superoxide radical scavenging assay

Antiradical activity procedure was in accordance with previously reported (83). The superoxide radical was generated by the NADH/PMS system, followed by reduction with NBT to form a formazan blue dye.

The reaction mixture consisted of 50 µL of sample, 50 µL NADH (166 µM), 150 µL NBT (43 µM) and 50 µL of PMS (2.7 µM). The plate reader was set in kinetic function, and the absorbance was determined for 2 min after PMS addition at 562 nm. All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were performed in triplicate.

1.5.3. Nitric oxide scavenging assay

$^{\cdot}$NO radical was generated by a SNP solution at pH 7.4 with posterior reaction with oxygen to produce nitrite, that was determined by Griess reagent (83).

To each well were added 100 µL of sample and SNP (20 mM), followed by 60 min of incubation at room temperature under light exposure. Finally 100 µL of Griess reagent

(1% sulfanilamide and 0.1% naphthyethylenediamine in 2% H_3PO_4) were added and the absorbance of the produced chromophore after the diazotization reaction at 540 nm was determined with a plate reader. Three experiments were performed in triplicate.

1.6. Cellular assays

1.6.1. Cell culture and treatments

Cells were maintained in DMEM with 10% FBS and 2% Pen-Strep in an incubator at 37 ^oC, with 5% CO_2 and controlled humidity, in 75 cm^2 flasks. Additionally Caco-2 cell medium was supplemented with amphotericin B (1%), transferrin (0.15% µL) and non-essential amino acids (1%).

Once confluence was achieved, cells were washed twice with 10 mL HBSS and 3 mL of trypsin-EDTA were added, followed by 8 min of incubation. 200 µL of the prepared cellular suspension (150000 cells/mL) was seeded in 96 well-plates and incubated for 3 days before carrying out the viability assays.

Preliminary assays were performed to determine the appropriate *t*-BHP concentration and exposure time to assess the antioxidant activity of the infusion (data not shown). Cells were seeded under the same conditions as described above. After 24 h, the medium was completely removed and *t*-BHP was added to the final concentration of 0.5 mM, with an exposure time of 6 h. MTT and lactate dehydrogenase (LDH) assays were then carried out to evaluate the effect of the infusion against the induced toxicity.

1.6.2. MTT assay

The yellow tetrazolium salt MTT is converted to an insoluble purple formazan product, by mitochondrial dehydrogenases of metabolically active cells.

Cells were incubated with different concentrations of the infusion for 24 h. After medium removal, 200 µL of MTT (0.5 mg/mL in DMEM) were added to each well, followed by 30 min of incubation. The extent of reduction to formazan was than quantified spectrophotometrically by measuring the absorbance at 510 nm, and compared to controls (84).

1.6.3. LDH assay

LDH is a cytosolic enzyme released into the culture medium after loss of integrity of the cellular membrane. LDH activity can be spectrophotometrically determined at 340 nm, by following NADH oxidation during the conversion of pyruvate to lactate (84).

The medium was collected after 24 h of cellular exposure to the infusion. The reaction mixture consisted of 50 µL of sample, 200 µL NADH (0.15 mg/mL) and 25 µL pyruvate (0.15 mg/mL), all prepared in phosphate buffer (0.1 M, pH 7.4).

1.6.4. Determination of total and oxidized glutathione

Total glutathione levels were determined by the DTNB-GSSG reductase recycling assay, after protein precipitation with perchloric acid (0.5%), and GSSG was determined after sample pre-treatment with 2-vinylpyridine for 1 h at 4 ºC, with agitation (85). The absorbance was read at 405 nm in kinetic function for 3 minutes. The experiments were conducted with and without exposure to *t*-BHP after incubation with the infusion. Proteins were quantified spectrophotometrically at 595 nm with Bradford reagent, using bovine serum albumin as standard.

1.6.5. Intracellular polyphenols' staining and fluorescence microscopy

The procedure was based on a previous work, with some modifications (86). Caco-2 and AGS cells were seeded at 75000 cells/mL, in 24-multiwell plates, and treated with infusion concentrations of 0.31 and 0.16 mg/mL respectively, for 24 h. Also a negative control and positive controls with apigenin (25 µM) and rosmarinic acid (25 µM) were evaluated under the same conditions.

Cell's fixation procedure consisted on removing the culture medium and adding 600 µL of the pre-warmed 3.7% formaldehyde solution, with 10 min of incubation at room temperature. The solution was then removed and each well was rinsed with 600 µL HBSS.

Nuclear staining was achieved with 1 µg/mL DAPI (with excitation at 364 nm and emission at 454 nm) for 30 min, followed by 0.1% (w/v) DPBA (excitation at 490 nm, emission at 530 nm) staining for 1 min. Fluorescence was detected by microscopy (Nikon

TS100 microscope, Tokyo, Japan) and digital images were generated with a Nikon DS-Fi1 camera and NIS-Elements D 3.2 software (Nikon Instruments INC, New York).

1.7. Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 Software (San Diego, CA, USA). Quantification of phenolic compounds was achieved from three determinations and results are shown as the mean (± SD). The EC_{50} values were calculated from three independent assays performed in triplicate. Cellular based assays consisted of six independent experiments and results are presented as mean values (± SEM). Data from different groups was compared using one-way ANOVA Tukey's multiple comparisons test. A level of statistical significance at $p<0.05$ was used.

Chapter IV - Results and discussion

1. HPLC-DAD-ESI/MS^n qualitative analysis of phenolic compounds

The HPLC-DAD-ESI/MS^n analysis of the aqueous extract of *M. pulegium* at 330 nm (Fig. 14) showed the presence of diverse compounds, the majority of them with an UV spectrum characteristic of cinnamoyl derivatives (**1-3**, **7**, **11-13** and **15**) (Table 4).

Compounds **1** and **3** exhibited the deprotonated molecular ion at *m/z* 353 (caffeoyl quinic acid) but different MS fragmentations, corresponding to 3-caffeoylquinic acid and 4-caffeoylquinic acid, respectively (Table 5), according to (87).

Compound **2**, with $[M-H]^-$ion at *m/z* 311 and MS fragmentation showing the loss of 132 amu (pentosyl moiety) originating the ion at *m/z* 179 ([caffeic acid-H]$^-$), can possibly be labelled as caffeoylpentoside. The MS^2 of compound **7** presented losses of -162 (hexosyl moiety), of -(162+18), corresponding to an interglycosidic linkage, and of -(162+132) (hexopentosyl moiety) over a caffeic acid (179, [caffeic acid-H]$^-$) (Table 4). Moreover, its MS^3[(M-H) →(M-H-162)]$^-$ was similar to the MS^2 of compound **2**; as so, compound **7** should be a derivative of compound **2** with an additional hexose over the pentose, being here characterized as a caffeoyl hexosylpentoside. Compound **12** exhibited an UV spectrum similar to that of **7.** Both the deprotonated molecular ion and the fragmentation pattern of compound **12** differed from those of compound **7** in 14 amu: since [feruloyl acid-H]$^-$ (193 amu) was observed instead of the ion at *m/z* 179 [caffeic acid-H]$^-$, compound **12** was identified as feruloyl hexosylpentoside.

With a similar UV spectrum (Table 5) and a deprotonated molecular ion at *m/z* 359, compound **11** presented in its MS^2 fragmentation losses of 3,4-dihydroxyphenyllactic acid (danshensu (DSS),-198 amu), caffeic acid (-180 amu) and caffeic acid-18 (-162 amu), with formation of ions at *m/z* 161, 179 and 197, respectively; according to (88) these are characteristics of dimers, trimmers and tetramers of caffeic acid. This fragmentation pattern is in agreement with the one reported for rosmarinic acid (89) and the co-elution of the extract with a standard allowed the characterization of compound **11** as rosmarinic acid.

Compound **15** also displayed a similar UV spectrum (Table 5) and its MS fragmentation showed a loss of 176 amu (feruloyl moiety), with formation of the ion at *m/z* 359. As the fragmentation (MS^3[535→359]$^-$) observed matched with the one of rosmarinic acid, compound **15** was tentatively identified as a feruloyl rosmarinic acid derivative.

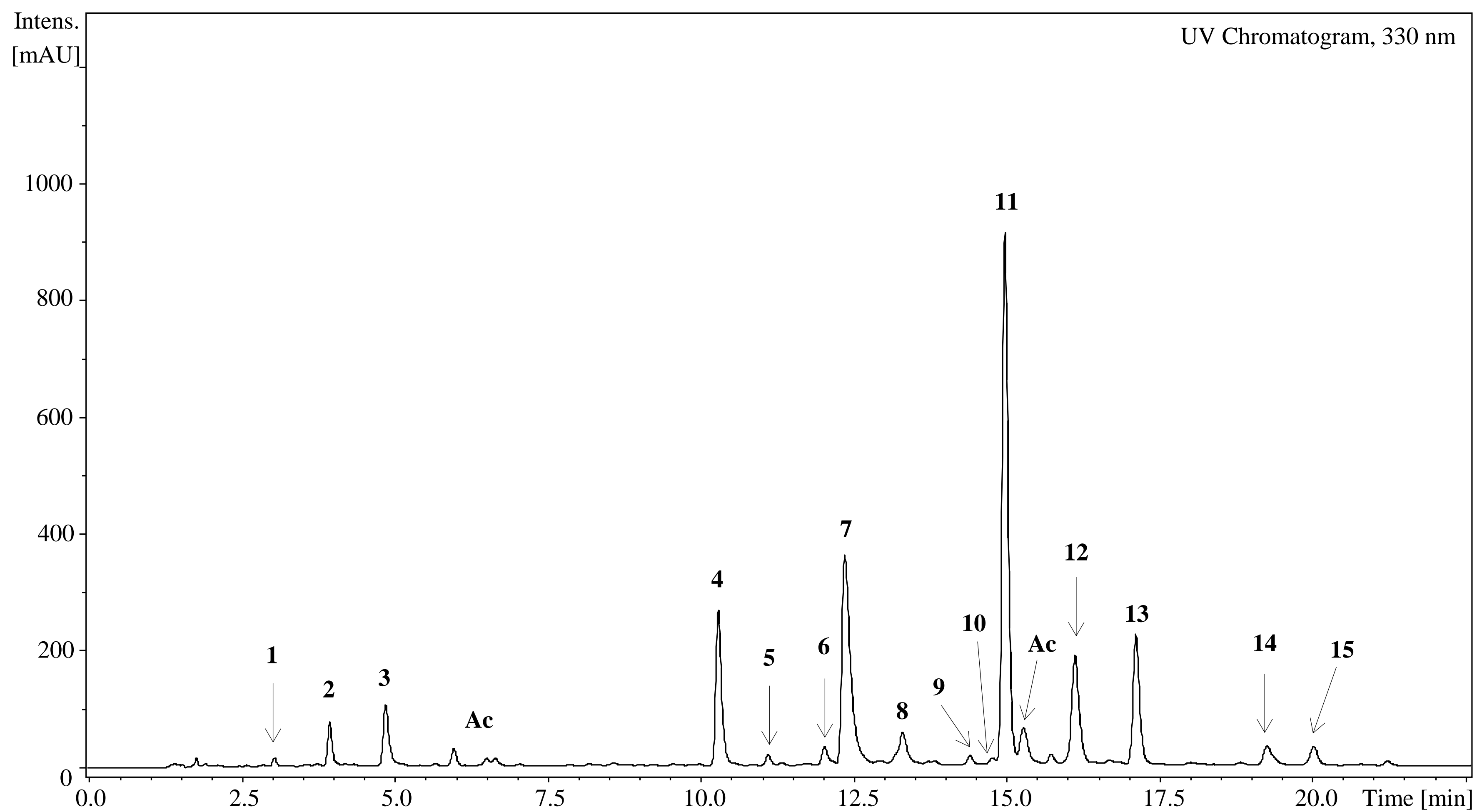


Figure 14. HPLC-DAD-ESI/MS^n chromatogram (330 nm) of *M. pulegium* infusion. Ac: not identified cinnamoyl derivatives. Identity of compounds as in Table 5.

Table 5. Rt, UV and MS: $[M-H]^-$, $MS^2[M-H]^-$ and $MS^3[(M-H)\rightarrow base\ peak]^-$ data of phenolic compounds from *M. pulegium* infusion [a].

	Compounds[b]	*R*t (min)	UV (nm)	$[M-H]^-$, *m/z*	$MS^2[M-H]^-$, *m/z* (%)	MS^3, *m/z* (%)
1	3-CaffQuin Ac	3.1	300sh, 328	353	191(100), 179(45)	
2	CaffPent	4.0	300sh, 328	311	179(65), 149(100)	
3	4-CaffQuin Ac	4.9	300sh, 328	353	173(100)	
4	Salv Ac H	10.3	252, 286, 314sh, 344	537	493(15), 339(100)	295(80), 229(100)
5	Lut-7-Rut	11.1	256, 266sh, 348	593	285(100)	
6	Lut-7-Gluc	12.0	256, 266sh, 348	461	285(100)	
7	CaffHexPent	12.3	300sh, 328	473	311(100), 293(60), 179(10)	179(50), 149(100)
8	Salv Ac E isomer	13.3	256, 284, 316, 348	717	519(100), 475(35)	475(100)
9	Chrys/Diosmt-7-Rut	14.4	256, 268sh, 348	607	299(100), 284(30)	
10	Apig-7-Gluc	14.8	267, 337	445	400(10), 269(100)	
11	Rosm Ac	15.0	300sh, 328	359	197(30), 179(40), 161(100)	
12	FerHexPent	16.1	300sh, 328	487	325(100), 307(57), 293(85), 193(30)	193(100), 149(4)
13	Lith Ac	17.1	298sh, 325	537	493(100), 359(35)[c]	359(100)
14	Salv Ac C isomer	19.2	262, 320	491	311(100)	
15	Fer Rosm Ac	20.0	300sh, 328	535	359(90), 177(100)[d]	

[a] Main observed fragments. Other ions were found but they have not been included.

[b] Ac: acid; Caff: caffeoyl; Fer: feruloyl; Quin: quinic; Pent: pentosyl; Hex: hexosyl; Gluc: glucuronoyl; Lut: luteolin; Apig: apigenin; Chrys: chrysoeriol; Diosmt: diosmetin; Salv: salvianolic; Rosm: rosmarinic; Lith: lithospermic; Rut: rutinoside.

[c] $MS^3(537\rightarrow359)$: 197(40), 179(40), 161(100).

[d] $MS^3(535\rightarrow359)$: 197(20), 179(35), 161(100).

Compounds **4**, **13** and **14** are trimmers. Compound **4** presented $[M-H]^-$ ion at *m/z* 537, losses of DSS (-198, *m/z* 339 base peak) and of a carbonyl group (-44) coincident with the fragmentation observed for salvianolic acid H (SAH) (87). In addition, its UV spectrum, different from those of the other caffeic acid derivatives above described, has already been reported (90). As for compound **13**, an isomer of compound **4**, an UV spectrum characteristic of caffeic acid derivatives was observed (Table 5). Both the similar MS fragmentation, showing a base peak corresponding to the loss of a carbonyl group (-44, *m/z* 493), and chromatographic mobility compared to rosmarinic acid were reported for lithospermic acid (88); therefore it was tentatively labelled as lithospermic acid. The last, compound **14**, exhibited the same deprotonated molecular ion of salvianolic acid C (*m/z* 491), also with the same chromatographic behavior, eluting at the end of the chromatogram. However, the loss of the DSS (-198) fragment with formation of the base peak reported before (88) was not observed; instead, the fragmentation originated the base peak at *m/z* 311 by loss of a caffeic acid moiety (-180), as reported in (90), either caused by different analytical conditions or by the presence of an isomer. Thus, it was tentatively labelled as salvianolic acid C isomer.

Compound **8** is a tetramer ($[M-H]^-$ at *m/z* 717). Its MS fragmentation showed the loss of DSS, giving rise to the base peak (*m/z* 519), as reported for the tetramers salvianolic acids E, B and L (91). Nevertheless, taking into account its lower chromatographic mobility, compared with that of rosmarinic acid, and strong resemblance with the fragmentation pattern and UV spectrum of a salvianolic acid E isomer described before (90), compound **8** may correspond to this molecule.

Other compounds with UV spectra of cinnamoyl derivatives were noticed at *R*t 6.0 and 15.3 min (**Ac**, Fig. 14), though their structures could not be determined.

Finally, four other peaks (**5**, **6**, **9** and **10**) found in trace amounts and with UV and MS spectra of flavonoids were observed. Compounds **5**, **6** and **9** presented an UV spectrum characteristic of flavones with di-substitution at the B ring (Table 5). The MS fragmentation of compounds **5** and **6** showed the ion at *m/z* 285 ($[luteolin-H]^-$) as base peak, thus being identified as luteolin derivatives. Compound **5** fragmentation, with loss of 308 amu (rhamnohexosyl moiety) and without ions characteristic of interglycosyl linkage cleavage, suggested the presence of a rhamnosyl(1→6)hexoside linkage. As so, it was tentatively labelled as luteolin 7-*O*-rhamnosyl(1→6)glucoside (luteolin 7-*O*-rutinoside). Moreover, the loss of 176 amu (glucuronoyl moiety) by fragmentation of compound **6** led to its identification as luteolin 7-*O*-glucuronide. As it happened for compound **5**, in the MS fragmentation of compound **9** it was observed the loss of 308 amu, resulting in the formation of the deprotonated aglycone ion (*m/z* 299), pointing to a methyl-luteolin,

probably chrysoeriol (5,7,4’-OH-3’-OMe-flavone, 3’-Me-luteolin) or diosmetin (5,7,3’-OH-4’-OMe-flavone, 4’-Me-luteolin). Thus, compound **9** was tentatively labelled as chrysoeriol/diosmetin 7-*O*-rutinoside. Compound **10** was identified as apigenin 7-*O*-glucuronide due to the UV spectrum characteristic of an apigenin derivative (Table 4) and because the fragmentation of its deprotonated molecular ion ($[M-H]^-$, 445) resulted in the deprotonated molecular ion of apigenin ($[apigenin-H]^-$, 269), the base peak, by loss of a 176 amu fragment (glucuronoyl moiety).

With the exceptions of rosmarinic and lithospermic acids and of diosmetin 7-*O*-rutinoside, all the other 12 compounds were identified for the first time in *M. pulegium* species.

2. HPLC-DAD quantitative analysis of phenolic compounds

The phenolic fraction of the infusion corresponded to 122.92 mg/g, distributed by the 15 identified compounds (Table 6).

Table 6. Phenolic composition of *M. pulegium* infusion.

Compounds[a]	mg/g lyophilized infusion[b]
1	4.16 ± 0.05
2	2.44 ± 0.02
3	2.64 ± 0.04
4	7.60 ± 0.76
5	0.64 ± 0.02
6	1.40 ± 0.06
7	16.20 ± 1.36
8	1.88 ± 0.12
9	3.40 ± 0.13
10	1.32 ± 0.09
11	66.08 ± 5.19
12	3.48 ± 0.10
13	7.52 ± 0.69
14	1.84 ± 0.03
15	2.24 ± 0.07
Σ[c]	122.92

[a] Identity of compounds as in Table 5.
[b] Results are expressed as means ± standard deviations of three independent determinations.
[c] Sum of the quantified compounds.

Phenolic acids represented 94% of the determined compounds. Rosmarinic acid (compound **11**) was the main compound (54% of the quantified compounds), as expected for a Lamiaceae species, being followed by other cinnamoyl derivatives, namely caffeoyl hexosylpentoside (compound **7**, 13%) and the trimmers salvianolic acid H (compound **4**) and lithospermic acid (compound **13**, 6%, each) (Table 6).

As for the identified flavonoids, chrysoeriol/diosmetin 7-*O*-rutinoside (compound **9**) was the most abundant one (3%).

3. Antiradical activity

The antiradical potential of *M. pulegium* infusion was evaluated by three different methodologies, as preliminary screening preceding the cellular assays (Fig. 15).

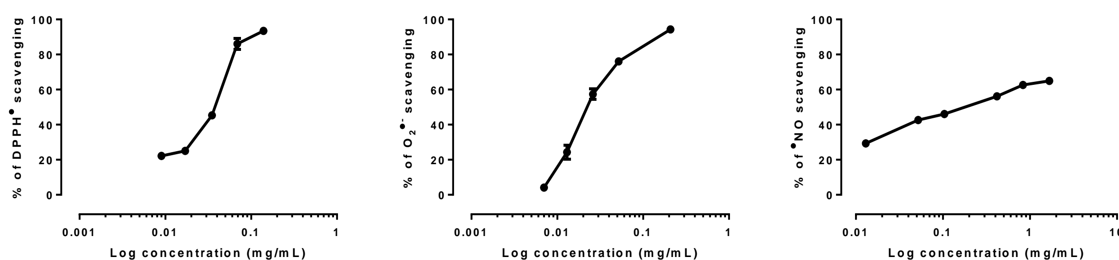


Figure 25. Antiradical activity of *M. pulegium* infusion against DPPH·, $O_2^{\cdot-}$ and ·NO radicals.

Concentration-dependent responses were observed, although total scavenging activity was not reached for ·NO radical. The EC_{50} values obtained were of 23, 39 and 226 µg/mL against DPPH·, $O_2^{\cdot-}$ and ·NO radicals, respectively.

A similar result was obtained in a previous work, in which the hot water extract revealed to be very effective against DPPH· (57). As for the other radicals, no previous results were found. As referred above, phenolic compounds are extremely effective in neutralizing radical species. Therefore, the results here obtained can be, at least partially, explained by the phenolic content of the tested infusion (Table 6).

Since ROS and RNS are directly and indirectly involved in some cellular oxidative stress mechanisms, dietary polyphenols intake may display an important role on balancing potential toxic environments.

4. Cellular viability and antioxidant protection against *t*-BHP induced toxicity

Originally, Caco-2 cells derive from human colon carcinoma and AGS cells from human gastric carcinoma. Both cell lines were selected for this study as models of cellular response to xenobiotics since, when consumed, *M. pulegium* infusion directly contacts with gastric and intestinal epithelia.

A preliminary experiment was conducted in order to assess the range of concentrations for which the exposure to the infusion was not able to affect cellular viability (Fig. 16).

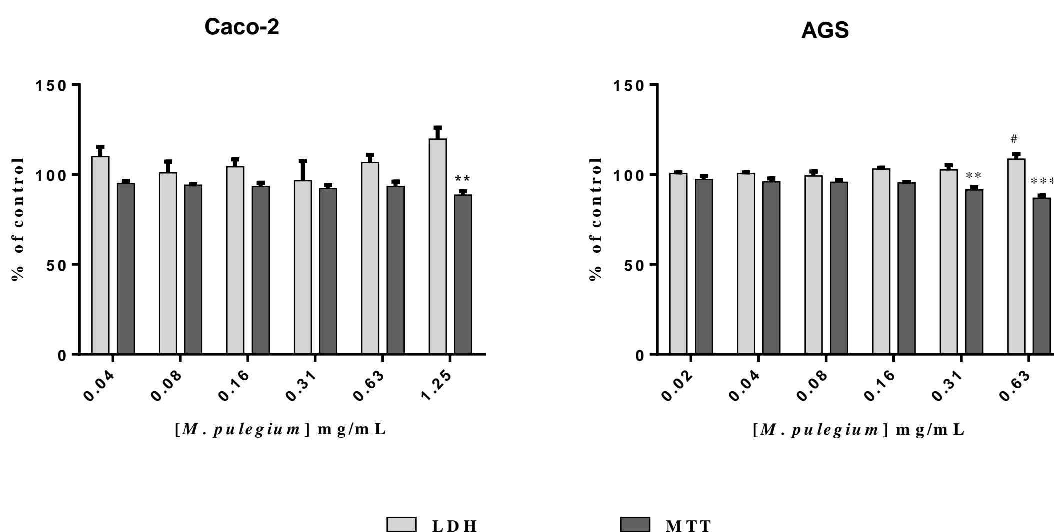


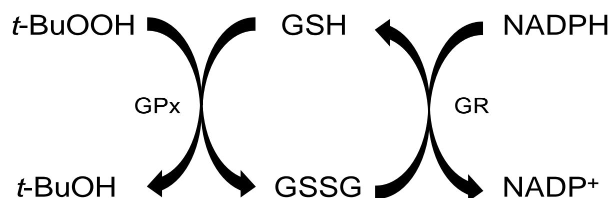
Figure 16. Effect of *M. pulegium* infusion on Caco-2 and AGS cell lines viability after 24 h of exposure, assessed by MTT reduction and LDH leakage assays. Values show mean ± SEM of six independent assays performed in triplicate (**p<0.01, ****p<0.0001 and #p<0.05 compared to the respective controls).

When comparing the two cell lines, AGS cells demonstrated to be more sensitive, since viability started to significantly decrease when exposed to 0.31 mg/mL of infusion, while for Caco-2 this happened only at 1.25 mg/mL (Fig .16). Moreover the results obtained by the MTT reduction assay were more expressive than the ones by LDH leakage for both cell lines, thus suggesting that mitochondrial damage happens prior to membrane's damage.

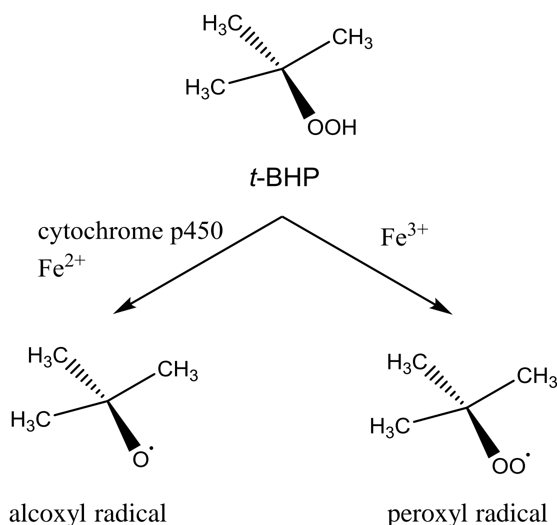
The following step was to evaluate the potential of *M. pulegium* infusion to protect the cells against the toxicity caused by *t*-BHP. This hydroperoxide is frequently applied to induce oxidative stress, the toxicity to hepatocytes being a result from either the metabolism through cytochrome p450 or reduction of GSH. As result, several biomarkers

can be monitored, like the increased levels of alanine transaminase, aspartate aminotransferase, LDH and malondialdehyde, and also GSH depletion (92).

t-BHP is reduced by GPx leading to the formation of *t*-butanol, with GSH depletion and increased GSSG:



Cytochrome p450 or Fe^{2+} and Fe^{3+} ions can also catalyze its transformation into alcoxyl and peroxyl radicals that can trigger lipid peroxidation process (92):



Additionally, alterations in the intracellular calcium homeostasis, metal chelation and DNA strand breaks are also some phenomena underlying its toxicity (92)

In this work, cells were treated with *M. pulegium* infusion for 24 h prior to *t*-BHP exposure (0.5 mM, 6 h). Cellular viability was again determined by MTT reduction and LDH leakage assays (Fig. 17).

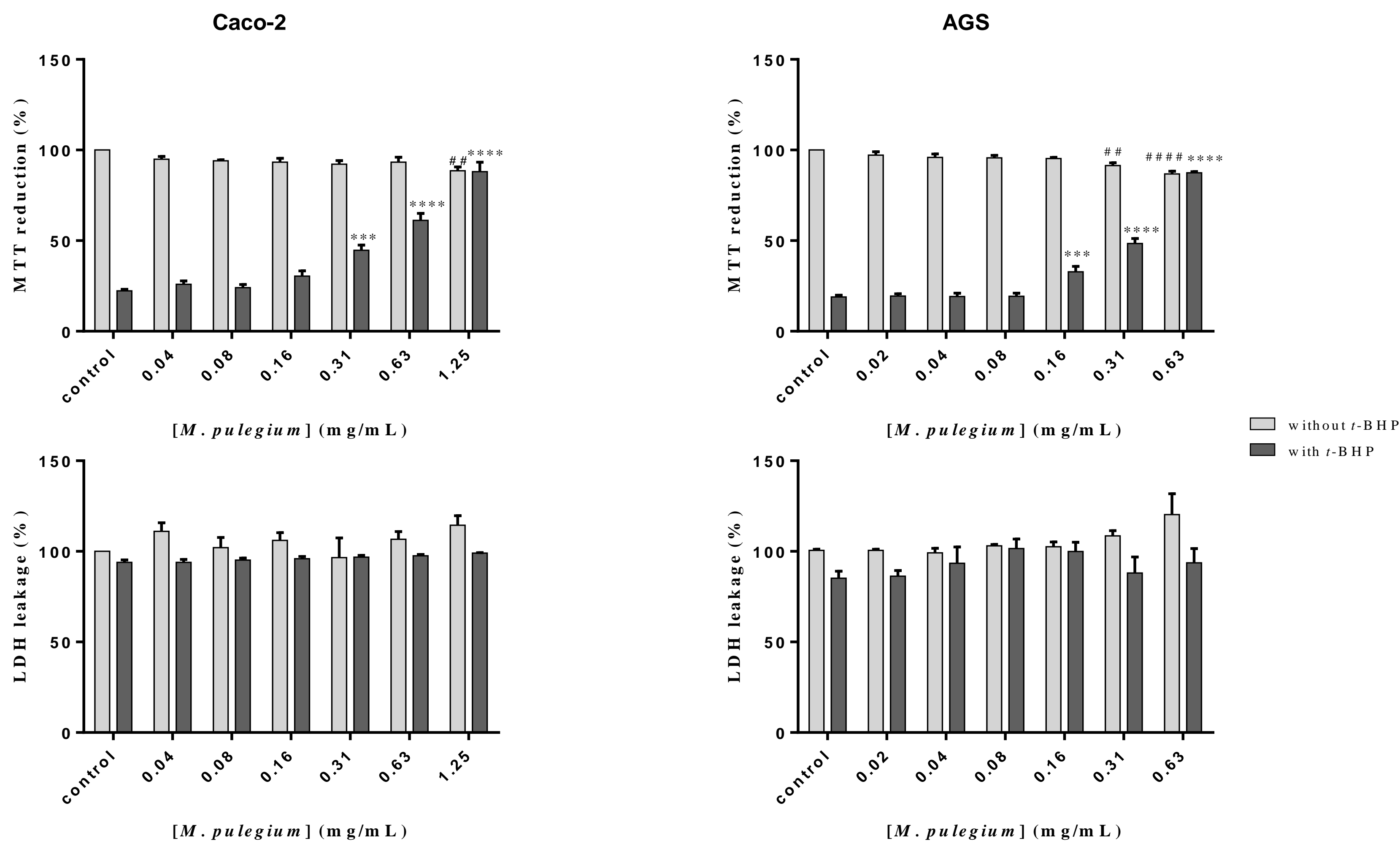


Figure 17. Caco-2 and AGS cellular viability assessed by MTT reduction and LDH leakage assays, after exposure to *M. pulegium infusion*, with and without t-BHP-induced toxicity. Cells were pre-treated with the infusion for 24 h. Insulted cells were further exposed to t-BHP (0.5 mM) for 6 h. , Values show mean ± SEM of six independent experiments performed in triplicate (***$p<0.001$, ****$p<0.0001$ and ##$p<0.01$, ####$p<0.0001$ compared to the respective controls).

The infusion clearly exerted a dose-dependent protective effect in the MTT reduction assay. In fact, total protection, when compared to controls, was almost achieved with the infusion at the concentrations of 1.25 and 0.63 mg/mL for Caco-2 and AGS cells, respectively (Fig. 17), despite that at this concentrations the infusion induced cytotoxicity by itself (Fig. 16). Hence, it can be assumed that from the pre-treatment with the infusion and post exposure to the toxicant did not result a synergic toxic effect.

Contrarily to what was expected, an increase of LDH leakage was not observed in neither cell lines (Fig. 17). In a previous work using LDH leakage as a biomarker for necrosis, and DNA fragmentation as a biomarker for apoptosis, it was reported that a concentration of *t*-BHP of 0.4-0.5 mM provides a transition point below which apoptosis is favored and beyond which necrosis is favored (93). Taking this into consideration, it can be inferred that for the experimented *t*-BHP exposure conditions (0.5 mM, 6 hours) cell death was triggered by apoptosis, thus not being observed the increase of extracellular LDH, which is related with a cellular membrane damage.

The phenolic composition of *M. pulegium* infusion here reported (Table 5) can be implicated in the antioxidant protective effect observed. Other flavonoids, like rutin and quercetin, also tested in a similar pre-treatment experimental model, were very effective at preventing Caco-2 DNA damage induced by *t*-BHP (94). The authors attributed such results to the metal iron chelating and free radical scavenging properties of those compounds. With HepG2 cells, but using a co-exposition model, it was demonstrated that other phenolics like luteolin, quercetin, luteolin 7-*O*-glucoside, caffeic and rosmarinic acids also protected the cells against *t*-BHP, by preventing lipid peroxidation and GSH depletion (95).

5. Total and oxidized GSH determination

Attempting to disclosure a possible mechanism behind the antioxidant activity observed, total glutathione (GSx) and GSSG levels were quantified under the same model of pre-treatment with the infusion, followed by *t*-BHP-induced toxicity (Fig. 18).

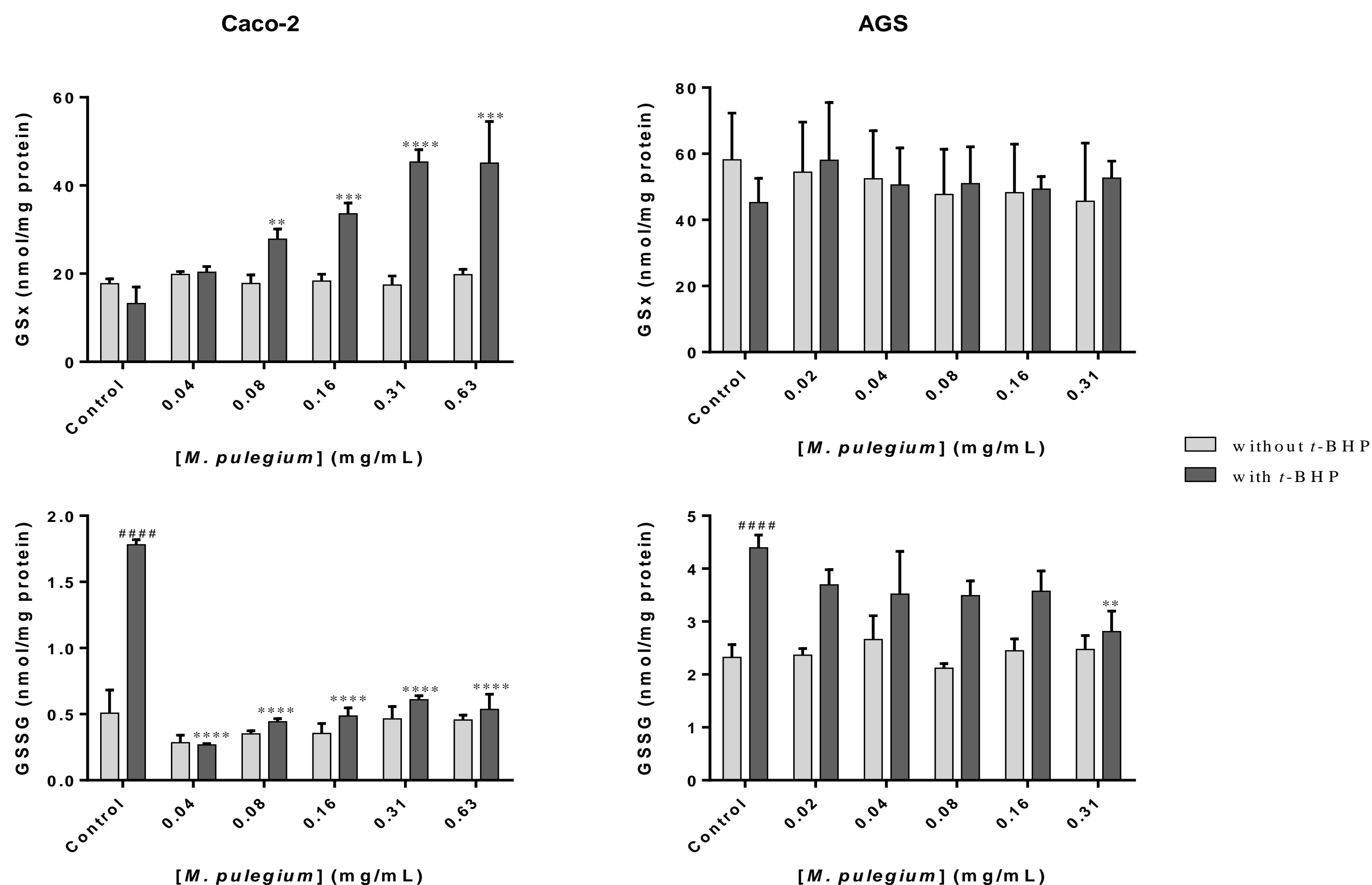


Figure 18. Total glutathione (GSx) and oxidized glutathione (GSSG) determination. Caco-2 and AGS cells were pre-treated with *M. pulegium* infusion for 24 h. GSx and GSSG levels were quantified with and without *t*-BHP exposure. Values show mean ± SEM of three independent assays performed in triplicate (**p<0.01***p<0.001 and ****p<0.0001 compared to respective controls and ####p<0.0001 comparison with and without *t*-BHP).

The results revealed a different response of the two cell lines. In Caco-2 cells, no increase of GSx levels was observed when compared to respective controls without infusion treatment. However, when post-exposed to *t*-BHP, GSx levels increased very significantly in a concentration-dependent response for infusion concentrations above 0.04 mg/mL (Fig. 18). This increase may be the result of a synergic effect over the ɣ-glutamylcysteine synthetase enzyme, the rate limiting enzyme responsible for glutathione synthesis, which is known to be more active in response to cellular treatments with phenolic compounds, leading to *de novo* synthesis (28). As expected, after *t*-BHP exposure higher GSSG levels were observed when compared to the respective control, and it was demonstrated that the infusion was able to prevent GSH oxidation (Fig. 18). Taking together these results with the ones obtained for the MTT reduction assay (Fig. 17), it can be noticed that the infusion prevented GSH oxidation even at concentrations at which it was not able to avoid cell death (0.04 to 0.16 mg/mL). As so, this mechanism is certainly important, but not the only one behind the protective effect observed.

In contrast, under the same experimental conditions, the infusion was not able to exert the same effect in AGS cells (Fig. 18). GSx levels were not significantly altered by neither the infusion nor the toxicant exposures. However, for the concentration of 0.31 mg/mL, a significant decrease of the GSSG levels was observed (Fig. 18), in accordance with the verified viability increase (Fig. 17). The results indicate that for AGS cells, *t*-BHP reduction by GSH was not the most relevant toxicity mechanism.

6. DPBA staining of intracellular polyphenols

DPBA is capable of binding to flavonoids and phenolic acids in reactive sites after the loss of an ethanolamine chain, with adduct formation and fluorescence emission (96). One of its applications is related to flavonoids staining in plant cells, to understand the mechanisms of their transport and to follow their localization (97).

To sustain if the phenolic compounds present in *M. pulegium* infusion were able to enter into the cells, a DPBA staining technique was applied. Nuclear staining with DAPI was also used to check on nuclear morphology. Based on the phenolic profile here reported, phenolic acids were the major represented class, rosmarinic acid being the one at the highest concentration (Table 5). So, in addition to the positive apigenin control (25 µM), as described in a previous work (98), a rosmarinic acid control (25 µM) was also tested.

The results showed that after 24 h of exposure to the infusion, the phenolic compounds could yet be targeted inside Caco-2 and AGS cells (Fig. 19).

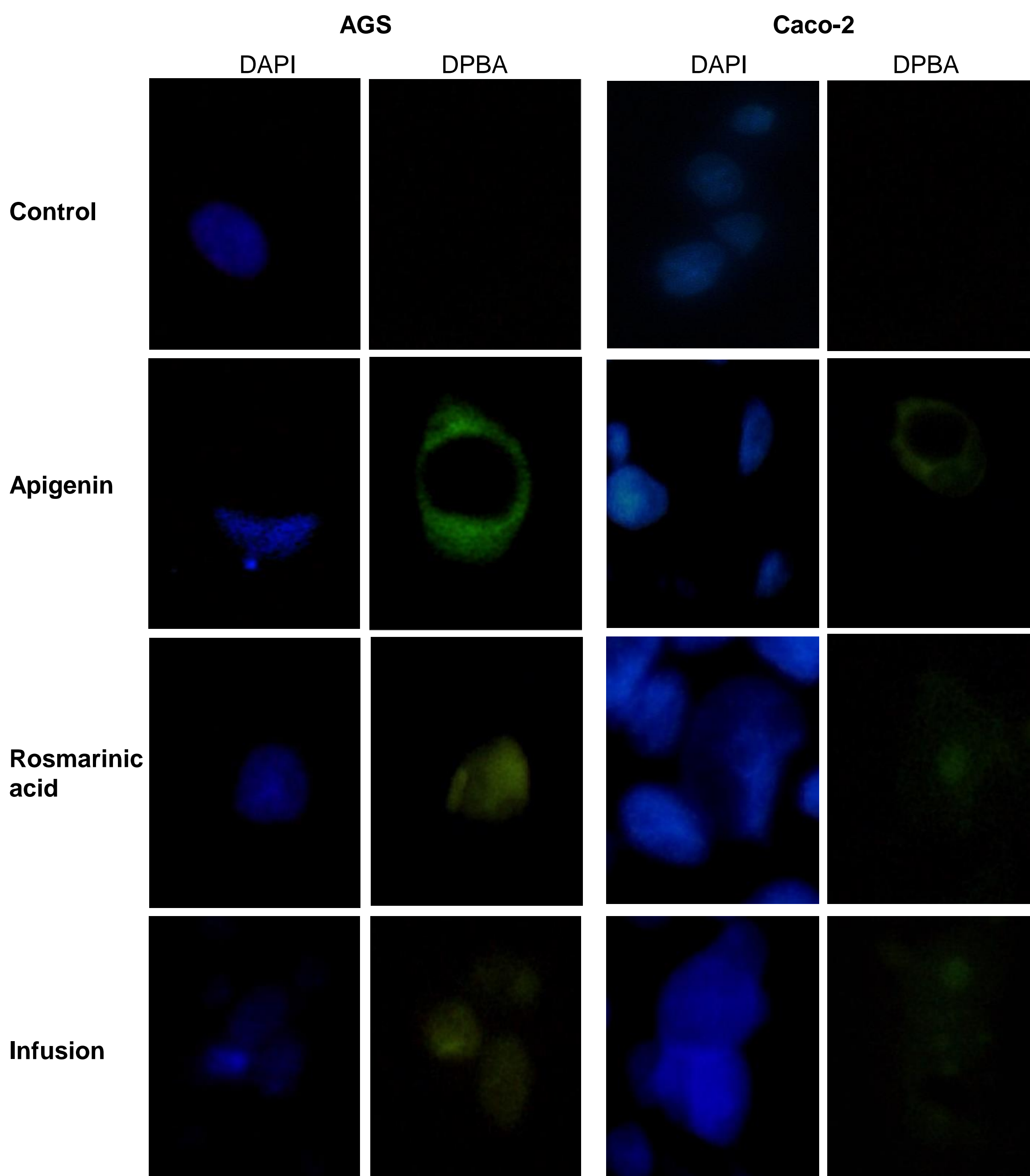


Figure 19. Fluorescence microscopy of intracellular DPBA staining of phenolic compounds and DAPI nuclear staining. Caco-2 and ÁGS cells were treated with *M. pulegium* infusion at the concentrations of 0.31 and 0.16 mg/mL, respectively, for 24 h. Three independent experiments were performed, and a representative field was selected.

Based on the positive controls, the detected fluorescence was derived by DPBA linkage to flavonoids and phenolic acids (Fig. 19). In agreement with the results reported in a previous work with leukemia cells (98), when incubated with apigenin, AGS and

Caco-2 cells seem to undergo an apoptotic process where chromatin condensation and vacuolization are clearly observed (Fig. 19).

As for rosmarinic acid controls, in comparison to the respective negative controls some slight morphological alterations can be observed, but in a smaller scale when compared to apigenin (Fig. 19). It has been reported that despite its antioxidant properties, rosmarinic acid can also act as antiproliferative and as an apoptotic inducer agent (99-101).

The viability assays did not demonstrate cellular toxic effects for the experimented infusion concentrations (Fig. 16). However, nuclear morphology alterations may be earlier visible than other toxicological end-points.

This works also sustained that the phenolic compounds were capable to pass through the cellular membrane, even without *prior* hydrolyses. More important, since the protective effect was observed under a pre-treatment model, the phenolic compounds were still inside the cells when exposed to *t*-BHP. Besides GSH defenses, phenolic compounds might also act directly over the toxicant.

As far as we know, no previous work focusing intracellular phenolic compounds staining and related antioxidant potential, of neither isolated compounds nor herbal extracts, in Caco-2 and AGS cells was performed.

Chapter V - Conclusions

With this work it was established for the first time the phenolic profile of *M. pulegium* infusion. Also, 12 new compounds were identified for the first time in this species. Rosmarinic acid was the major compound. The chemical profile found supports the very effective scavenging activity against $DPPH^{\cdot}$, $^{\cdot}NO$ and $O_2^{\cdot -}$ radicals. The infusion demonstrated to protect Caco-2 and AGS cells against *t*-BHP-induced toxicity, in a concentration-dependent manner, probably due to GSH induction and prevention of GSSG formation. It was also demonstrated that, even being glycosylated, the phenolic compounds were able to pass through the cellular membrane and act as direct antioxidants.

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