UNIVERSITY OF SALAMANCA

FACULTY OF PHARMACY

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

Phenolic composition and health-promoting effects of *Salvia* and *Thymus* decoctions

DOCTORAL THESIS

Andrea Luísa Fernandes Afonso

2019

UNIVERSITY OF SALAMANCA

FACULTY OF PHARMACY

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY

Phenolic composition and health-promoting effects of *Salvia* and *Thymus* decoctions

Doctoral Dissertation presented by **Andrea Luísa Fernandes Afonso**
for PhD degree of University of Salamanca

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Salamanca, 19th July

The results obtained in this Doctoral Thesis have been published (or accepted for publication) in international journals. Part of the information presented in this section was published in two chapters.

1. Olívia R. Pereira; Andrea F. Afonso; Susana M. Cardoso (2016). **"Advances in Analysis of Phenolic Compounds of *Salvia*, *Thymus* and *Lavandula* Plants".** In Govil, JN & Manohar Pathak, Recent Progress in Medicinal Plants Series, Vol 44: Phytotherapeutics III, Studium Press LLC, USA, Chapter 5. pp 75-108. Series ISBN 0-9656038-5-7, ISBN 1-62699-081-6.

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2. Andrea F. Afonso; Jorge M. Alves-Silva; Olívia R. Pereira; Susana M. Cardoso (2016). **"Beneficial Effects of *Salvia* Plants: Correlation with Bioactive Components"**. In Govil, JN & Manohar Pathak, Recent Progress in Medicinal Plants Series, Vol 44: Phytotherapeutics III, Studium Press LLC, USA, Chapter 8, pp 161-198. Series ISBN 0-9656038-5-7, ISBN 1-62699-081-6.

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3. Andrea F. Afonso, Olívia R. Pereira, Rodrigo T. Neto, Artur M. S. Silva, Susana M. Cardoso. **"Health-Promoting Effects of *Thymus herba-barona, Thymus pseudolanuginosus* and *Thymus caespititius* Decoctions",** *International Journal of Molecular Sciences*, Volume 18, 2017. DOI:10.3390 / ijms18091879.

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4. Andrea F. Afonso, Olívia R. Pereira, Mónica Válega, Artur M. S. Silva, Susana M. Cardoso. **"Metabolites and Biological Activities of *Thymus zygis, Thymus pulegioides* and *Thymus fragrantissimus* Grown under Organic Cultivation"**, *Molecules*, Volume 23, 2018. DOI:10.3390 / molecules23071514.

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5. Olívia R. Pereira, Marcelo D. Catarino, Andrea F. Afonso, Artur M. S. Silva, Susana M. Cardoso. **"*Salvia elegans, Salvia greggii* and *Salvia officinalis* Decoctions: Antioxidant Activities and Inhibition of Carbohydrate and Lipid Metabolic Enzymes"**, *Molecules*, Volume 23, 2018. DOI:10.3390 / molecules23123169.

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6. Andrea F. Afonso, Olívia R. Pereira, Ângela S. F. Fernandes, Ricardo C. Calhelha, Artur M. S. Silva, Isabel C.F.R. Ferreira, Susana M. Cardoso. **"Health-benefits and phytochemical profile of *Salvia apiana* and *Salvia farinacea* var. *victoria blue* decoctions"**, *Antioxidants*, accepted for publication (manuscript ID: antioxidants-545208).

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Fdo. Susana M. Cardoso

Fdo. Olívia R. Pereira

ACKNOWLEDGEMENTS

This PhD thesis was only possible with the help and friendship of many people and Institutions, so I am very grateful to all.

First I would like to express my gratitude to my supervisors, Doctor Susana M. Cardoso and Doctor Olívia R. Pereira, whom I had the honor and the pleasure of working. I want to thank for her encouragement, good ideas and friendship. I am also thankful for all the teaching, support, for all help and follow-up in the development of this work. Thanks for showed me and advised me to overcome my weaknesses and so to get here.

I want to express my thanks to Doctor Mª Ángeles Serrano García, for her support, for her valuable advices and for the opportunity to extend the date of the defense of this thesis. I also would like to thanks to my tutor, Doctor José Juan García Marín, for giving me the opportunity to performed this doctoral thesis, for all the help in correcting the Castilian text, and suggestions in this work.

I also would like to extend my appreciation and gratitude to Doctor Rocío I. Rodríguez Macías for her encouragement, availability and clarification of doubts and kind words during this period.

I address my warm thanks to Doctor Artur M. Silva, for giving me the possibility to join his investigation group and for their valuable guidance. I also thank the collaboration of Doctor Mónica Valéga, Doctor Rodrigo Neto and Doctor Marcelo Catarino, for their contribution in antioxidant and anti-inflammatory experiments of *Thymus* plants.

I thank the collaboration of Doctor Isabel Ferreira for welcoming me in her laboratory where I was granted the privilege of working. I also thank to Doctor Lillian Barros, Doctor Ângela Fernandes and Doctor Ricardo Calhelha for their valuable contribution in antioxidant, anti-inflammatory and cytotoxicity experiments of *Salvia* plants, respectively.

Furthermore, I want to express my thanks to my colleagues and friends of the Health School of Bragança - Polytechnique Institute of Bragança, for the friendship during this journey that was long and hard, specially thanks to Vera Lebres for her empathy and for the encouragement to finish this work.

I address my warm thanks to all staff of Public Health Laboratory where I work and where the antimicrobial experiments were developed. Particularly thanks to my colleagues and friends Helena and Gena, with whom I work daily, for the support, companionship and understanding manifested during this period.

I address my warm thanks to my friends Bebiana and Bruno Pires, for the support and advice but principally for positive energy and friendship that has always given me.

Lastly, I would like to thank my family of the heart for always believing in me. In particular, my husband António José for all his strength, support, courage and love without it nothing was possible. My parents and my sisters Célia and Cláudia for the encouragement, for all their absolute support and genuine love.

I dedicate my work to all my family in particular to my son Mateus that was born during this investigations, becoming my main source of inspiration.

Thank you all.

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LIST OF ABBREVIATIONS

AAE	Ascorbic acid equivalent
ABTS	2,2’-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole (2(3)-t-butyl-4-hydroxyanisole)
BHT	Butylated hydroxytoluene (3.5-di-tert-butyl-4 hydroxytoluene)
CAT	Catalase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DAD	Diode array detection
DW	Dry weight
DMNQ	2,3-dimethoxy-1,4-naphthoquinone
DNA	Deoxyribonucleic acid
DPPH$^{\bullet}$	Radical 2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
ESI	Electrospray ionization
FRAP	Reducing/antioxidant power
GAE	Gallic acid equivalents
GPx	Glutathione peroxidase
GSH	Reduced glutathione
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Half maximal inhibition concentration
IL	Interleukin
INF	Interferon
iNOS	Inducible nitric oxide synthase
LC	Liquid chromatography
LD	Detection limit
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LQ	Quantification limit
MAPK	Mitogen-activated protein kinases
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration

MMC	Minimum microbicidal concentrations
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS^n	Tandem mass spectrometry
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulphophenyl)-2H-tetrazolium inner salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MUFA	Monounsaturated fatty acids
NCTC	National Collection of Type Cultures
Nf-kB	Nuclear factor-κB
$NO^•$	Nitric oxide radical
$O_2^{•-}$	Superoxide radical
$OH^•$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
ORAC	Oxygen radical absorbance capacity
PAMP	Pathogen associated molecular pattern
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinase
PRR	Pathogen-recognition receptor
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Reducing power
RT	Retention time
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SFA	Saturated fatty acids
SOD	Superoxide dismutase
SRB	Sulforhodamine B
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalence antioxidant capacity
TGF-β	Transforming growth factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alfa
UFA	Unsaturated fatty acids
UHPLC	Ultra high performance liquid chromatography
UTAD	University of Trás-os-Montes e Alto Douro
UV	Ultraviolet
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

1. INTRODUCTION

In recent years, several beneficial activities of plants have been attributed to their polyphenolic composition [1,2]. On the other hand, Mediterranean region is rich in medicinal plants, both in wild or cultivated forms, that because of their medicinal value are potential candidates for exploitation by various industries, including the food and farmaceutic.

This first section of the Doctoral Thesis aimed to introduce the subject of the present work. For that, a general description of *Thymus* and *Salvia* genus together with the target plant species is made, followed by an overview of their characteristic phenolic constituents. Moreover, the potential health effects of these genera reported in the literature up to this moment, are summarized.

Part of the information presented in this section was published in the chapters “Advances in analysis of phenolic compounds of *Salvia, Thymus* and *Lavandula* plants” and "Beneficial effects of *Salvia* plants: correlation with bioactive components" which was published *in* "Govil, JN & Manohar Pathak, Recent Progress in Medicinal Plants Series", 2016, Vol 44: Phytotherapeutics III, Studium Press LLC, USA, Chapter 5. pp 75-108 and Chapter 8, pp 161-198, respectively.

1.1. General description of the plants

1.1.2 *Salvia* genus

Salvia is the largest genus in the Lamiaceae family plants, with over 900 species [3]. The name *Salvia* has its origin in the Latin word *Salvare,* that means "healer", in reference to the curative properties of these plant, which has been used since ancient times as a medicinal herb [4,5]. The plants of this genus are distributed worldwide, although they are particular prevalent in tropical and temperate regions of Europa around the Mediterranean area, in South-East Asia and in Central and South America [3,6]. *Salvia* plants are mostly aromatic and perennial shrubs, with flowers of distinct colors (e.g. violet, blue, lilac or pale-blue), size and structure, such as their staminal lever, the length and form of the corolla tube, and floral nectary morphology. In general, stems are long, reaching between 50 to 100 cm, with 3 to 5 branches. These contain opposite, simple, ovate and petiolate leaves [7–9].
Sage have been used for centuries in culinary, in cosmetic, in fragrance industry and in traditional medicine [4,5]. The latter application includes their use on a wide range of ailments, including digestive, respiratory, renal, hepatic, neurological, cardiac, blood circulation and metabolic, among others [10–13]. In these sense, many species of genus *Salvia* have been recognized to have potential uses in medical procedures and applications in pharmaceutical and cosmetic industries because their biological properties [1,14], mainly focused in its essential oils [15–18]. However, several researchers found that polar extracts, rich in phenolic compounds particularly phenolic acids and flavonoids, are also related with their biological properties, such as antimicrobial, antioxidant, anti-inflammatory, hypoglycaemic and others [2,19,20].

This research aims to evaluate the phenolic compounds of aqueous extracts of eight sage species: *Salvia officinalis,* the cultivar *Salvia officinalis 'Icterina', Salvia elegans, Salvia greggii, Salvia farinacea* var. *victoria blue, Salvia apiana, Salvia africana* and *Salvia mexicana* (Figure 1). Additionaly, the antioxidant, anti-inflammatory, antitumoral and antibacterial properties of the extracts will be screened, in order to evaluate their potency for the development of novel functional foods and/or therapeutic ingredients for the synthesis of new generation and alternative chemical drugs with low toxicity [2,21,22]

S. officinalis, also known as Dalmatian sage, is one of the most studied species. It is cultivated in Europe as a culinary herb or as an ornamental plant, where it was possibly introduced by Romans in ancient times or monks during the middle-ages by having a historical reputation of promoting health and treating ailments [4,23]. This specie has several cultivars, including ***S. officinalis 'Icterina'*** which is a cultivar characterized by

its yellow green variegated leaves [24]. In turn, the remaining *Salvia* species focused in the present study remain less-distributed and poorly studied regardless being used in culinary and/or traditional medicine. In particular, ***S. greggii***, commonly known as ''autumn sage'', is a biennial plant originating in both Mexico and Texas (USA) [25], although it is currently spread in southwestern United States and Arizona and cultivated in some parts of the world. It grows as a soft, evergreen shrub taller with about 1.2 m, and similarly to *S. officinalis*, its leaves are green and smoothie [26]. Its flowers, which appear between spring to autumn, can be of different colors, usually are red but may also appear as pink, purple, white or orange, and are characterized by an intense aroma and abundant néctar [27].

Figure 1 – *Salvia* species. A, B and C: Jardim Botânico da UTAD Retrieved from https://jb.utad.pt/jardim; D and E: HooksgreenHerbs Retrieved from https://www.hooksgreenherbs.com; F, G and H: Consulta Plantas Retrieved from http://www.consultaplantas.com.

Additionally, ***S. elegans*** is known as pineapple sage because its leaves smell like pineapple and can be used as a flavoring in food. In Mexico, this species is popularly known as "mirto" and has been widely used in mexican traditional medicine for treating central nervous system disorders, affording to treat headache and high blood pressure

[28,29]. In turn, ***S. apiana***, also recognized as white sage, is a branched shrub growing up to 1.5 m, with silvery white leaves highly aromatic, stems hollow, and white to pale lavender flowers, attractive to pollinators as honeybees (bee sage) [30,31]. This species is native to California, where some tribes consider it to be sacred and medicinal, and in addition is used in cosmetic applications and natural food additive [30,32]. Moreover, ***S. farinacea* var. *victoria blue***, known as mealy cup sage, is a perennial shrub typically growing 45-90 cm, native to Mexico and Texas [9], whose gray-green leaves are drooping, irregularly-serrate, ovate-lanceolate and it contains a compact multi-branched rich violet-blue flowers spikes, resembling lavender [9,33,34]. On another hand, ***S. mexicana***, also known as "Limelight", is an herbaceous perennial native to a wide area of central Mexico, that has large calyces bright lime green, dark leaves and purple-blue flowers not more than 4 cm long, visited by pollinators [35,36]. In addition, ***S. africana*** L., is an aromatic, hardy shrub up to 2 m in height, originating from Africa, whose essential oils composition was been studied [18,37,38].

1.1.1. *Thymus* genus

Thymus is a Latin name of plant genus, which is considered to derive from the Greek word *thyo* (perfume) by some authors, while others consider the Greek word *thymos* (courage, strength) [39]. *Thymus* genus belongs to Lamiaceae family and includes about 350 aromatic species that are distributed around the world, being particularly abundant in the west Mediterranean region [40,41]. These species are perennial and are characterized as being herbaceous subshrubs or shrubs with 10 to 30 cm tall, containing small and simple leaves, ramified and prostrated branches and big clusters of pink, white, cream or violet flowers [40].

Thymus species are recognized as being strongly aromatic and thus are widely used as spices to enhance sensory attributes, flavouring and improving the preservation of foods [42]. Because of these properties, compounds of thyme species have been investigated for the development of nutraceuticals and functional foods as health promoting ingredients [40,43,44]. The beneficial properties of thyme species include antimicrobial, antioxidant, anti-inflammatory, cardioprotective, neuroprotective, anticarcinogenic and hypoglycaemic activities, among others [1,45–47]. Although these applications have been mostly associated with essential oils [42,48,49], nowadays, *Thymus* polar extracts are an attractive target for the screening of health-benefits compounds for possible industrial applications in food, cosmetics or pharmaceutical industries [1,2].

Amongst *Thymus* plants, *Thymus vulgaris* is the most widespread worldwide and also the most studied. Other common species include *Thymus serpyllum* and *Thymus*

capitatus, among others [43,50]. In this context, it is the main aim of this this work to evaluate the phenolic composition and the possible biological properties of decoctions from six less-spread thyme species, namely *Thymus pulegioides, Thymus zygis, Thymus caespititius, Thymus herba-barona, Thymus fragrantissimus* and *Thymus pseudolanuginosus* (Figure 2).

Wild populations of ***T. pulegioides*** are known to exist in regions as distanct as Canadá and New Zealand [39], and in Europe, as well [50]. In Portugal, *T. pulegioides* takes the vulgar name Pojinha, large thyme or broad-leaved thyme, and is mainly found in the meadows of north of Portugal, particularly in Trás-os-Montes, where it is highly prized in folk therapy due to its claimed antiseptic and anti-inflammatory properties [50].

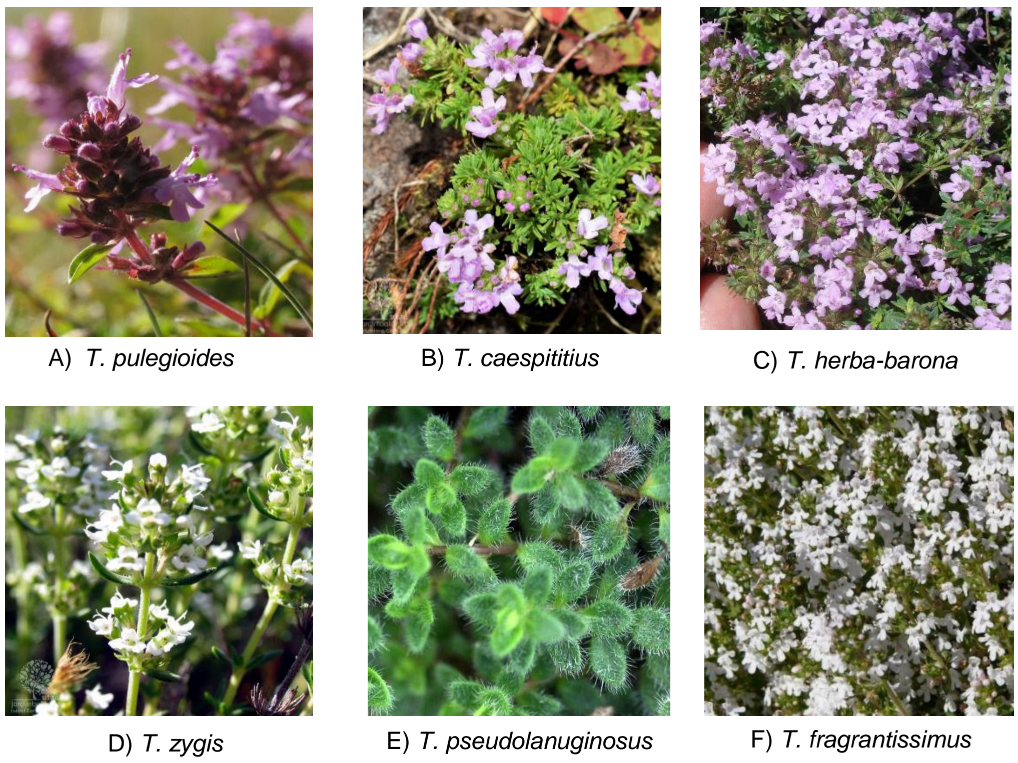


Figure 2 – *Thymus* species. A, B, C and D: Jardim Botânico da UTAD Retrieved from https://jb.utad.pt/jardim; E and F: HooksgreenHerbs Retrieved from https://www.hooksgreenherbs.com.

On another hand, ***T. caespititius***, is an Ibero-Macaronesian specie, whose geographical distribution seems to be very old, occurring in the northwest of the Iberian Peninsula and also in Madeira and Azores. *T. caespititius* differs from other species of *Thymus* because the plants have very long stems and also flat, non-revolute and glabrous leaves [39]. In addition, ***T. herba-barona***, also know as caraway thyme, is endemic in Mediterranean islands, particullarly in Majorca, Corsica and Sardinia, occurring in mountain areas at high altitudes [51]. It is used in folk medicine as a diaphoretic, sedative, antiseptic and

antimycotic and for the treatment of insomnia and is also utilized in perfumery and cosmetics. The bioactivity of this thyme has been extensively reported and associated mostly with its composition in essential oils [52,53]. Addictionaly, ***T. zygis***, also known as red thyme, is a small aromatic widespread endemic plant in the Mediterranean and is commonly used as a spice in several foods, as well as a remedy for whooping cough, bronchitis and rheumatism, mainly due to its claimed anti-inflammatory properties [42,54]. In turn, ***T. pseudolanuginosus***, commonly called as woolly thyme because of its characteristic hairs stems, is a perennial ground plant that is used to cover the soil. The leaves have little, if any fragrance, and are unsuitable for culinary use [55]. Moreover, ***T. fragrantissimus*** is also called oragne thyme because it has grey-green and orange scented leaves. Because of its orange aroma, it is widely used as culinary ingredient in soups, fish, meat, salads and vegetables cook dishes, albeit it is also claimed to have medicinal properties, sore throats, cough, mouthwash and for skin infections [56].

1.2. Phenolic compounds in *Thymus* and *Salvia* species

Previous studies on the chemical composition of *Thymus* and *Salvia* species have mainly dealt with their essential oils [42,48,57]. However in recent years, researchers have focused on other secondary metabolites, particularly the phenolic compounds [40,58]. These compounds, ubiquitous in plants are an essential part of the human diet, and are of considerable interest due to their health properties [2,20,47].

A detailed overview on the phenolic compounds of *Thymus* and *Salvia* genera is described in this section. Data is summarized in Tables 1, 2, 3 and 4 and the structure of polyphenols is depicted in Figures 4, 5, 6, 7, 8 and 9.

“Phenolic compounds” is a generic term that refers to a large number of compounds: approximately 8000 different phenolics dispersed throughout the plant kingdom. Chemically, they are organic compounds formed by at least one phenol ring, in which the hydrogen is usually replaced by a more active residue, such as hydroxyl, methyl or acetyl. They occur in different chemical structures, from simple phenolic molecules to complex high-molecular weight polymers, synthesized in plants through the pentose phosphate, shikimate and phenylpropanoid pathways [59–61]. The variable biological properties of the phenolics result from these structural diversity, and usually they contain more than one phenolic ring, and thus are often referred to as polyphenols [59,62].

Polyphenols can be divided into different groups according to their number of phenol rings tand on the basis of structural elements that bind these rings, and they are commonly found conjugated to sugars and organic acids. The most common classification of phenolic metabolites distinguishes the flavonoid and non-flavonoid compounds [59,60,62–64]. The chemical structure of non-flavonoid metabolites consists of the following subgroups: phenolic acids (hydroxycinnamic acids and hydroxybenzoic acids), lignans, stilbenes, tannins and lignins. On the other hand, flavonoid compounds group are divided into six main subclasses: flavonols, flavones, flavanones, flavan-3-ols, isoflavones and anthocyanidins [59,62–64]. The classification of phenolic compounds is presented in Figure 3.

1.2.1. Non-flavonoid compounds

Phenolic acids comprise important compounds that belong to non-flavonoid group, and are frequently divided in two subgroups, the hydroxycinnamic and hydroxybenzoic acids (Figure 3). Hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C6–C3) (Figure 4A), and include caffeic acid, chlorogenic acid, *o*-, *m*- and *p*-coumaric acid, ferulic acid and sinapic acid.

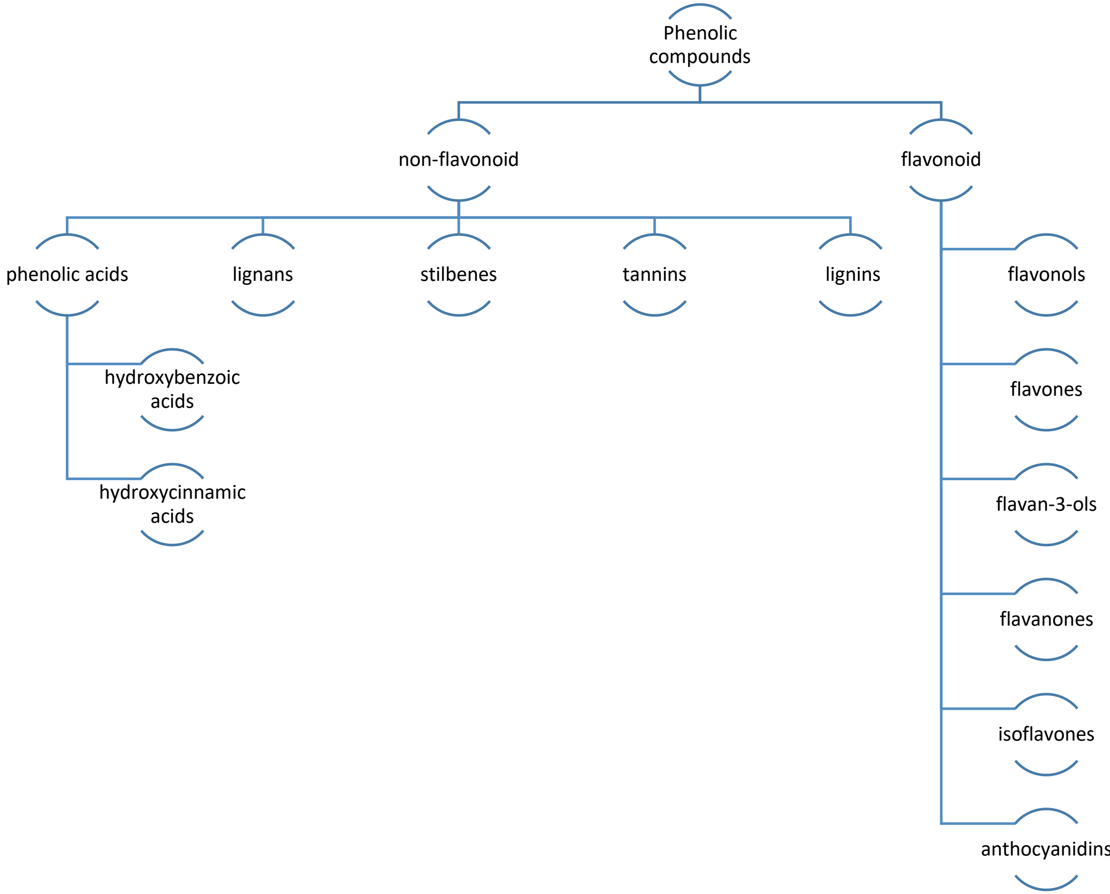


Figure 3 - The general classification of phenolic compounds

As products of the phenylpropanoid pathway, hydroxycinnamic acids have a basic structure of trans-phenyl-3-propenoic acid, with one or more hydroxyl groups attached to the phenyl moiety (Figure 4A). Instead, hydroxybenzoic acids are phenolic acids composed of simple phenols, also known as hydroxybenzoates, and include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the general C6–C1 structure (Figure 4B). The plant hydroxybenzoic acids occur mostly in the glycoside form [59,62,63]. Lamiaceae plants and, in particular, *Salvia* and *Thymus* genera, are mostly enriched in hydroxycinnamic acids, although others also occur [40,65]. The occurrence of non-flavonoid phenolics in the two plants genus is summarized in Table 1.

COOH HO A

COOH HO B

Figure 4 – General structure of hydroxycinnamic (A) and hydroxybenzoic (B) acids.

1.2.1.1. Hydroxycinnamic acid

Amongst hydroxycinnamic acids, **rosmarinic acid (1)**, an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid, is a major phenolic component in *Salvia* and *Thymus* plants (Table 1). The content of this caffeic acid ester in *S. officinalis* is quite variable (1.2 to 22 mg/g dry plant) [66–69]. Additionaly, this acid was described as a major phenolic constituent in methanolic extracts of *Salvia tomentosa* (10.2 mg/g dry plant) [70], *Salvia cadmica* (6.5 mg/g dry plant) [71], *Salvia fruticosa* (4.5 mg/g dry plant) [72], *Salvia verbenaca, Salvia argentea* and *Salvia aegyptiaca* (12.8, 4.8 and 6.0 mg/g dry plant, respectively) [66]. Likewise, it represents the most frequent detected compound in other *Salvia* plants, including *Salvia nemorosa* [73], *Salvia sclarea* L. [74], *Salvia fruticosa, Salvia pomifera* [75], *Salvia halophila* [76] and others [77].

Rosmarinic acid was also found in methanolic and aqueous extracts of *T. vulgaris*, presenting values in the range of 0.7 - 21.9 mg/g dry plant [68,78,79]. In addition, *T. serpyllum* (wild thyme) presented high amounts of this caffeic acid, ranging from 21.7 [80] to 57.7 mg/g dry plant [79]. In the recent years, others thyme species have been studied for phenolic characterization, presenting variable rosmarinic acid content: 1.7 to 43 mg/g dry plant in different populations of *Thymus mastichina* [81], 15.2 mg/g dry plant in methanolic extracts of *Thymus praecox* subsp. *grossheimii* var. *grossheimii* [82], 20.6 mg/g dry plant of methanolic extract of *Thymus nummularius* [83], 3.3 and 0.7 mg/g dry plant in methanolic and aqueous extracts of *T. capitatus,* respectively [84]. Additionaly, rosmarinic acid was found in high amounts in the methanolic extracts of *Thymus glabrescens*, *T. pulegioides* and *Thymus pannonicus* with concentratiosn between 0.8 and 1.4 mg/g dry plant [85].

Salvia and *Thymus* plants also contain distinct **rosmarinic acid derivatives**. In particular, extracts of *Salvia miltiorrhiza* and *T. serpyllum* were reported to contain **rosmarinic acid glucoside (2)** [80,86], while **(caffeoyl)rosmarinic acid (3)** was described to occur in *T. vulgaris* [87] and in *Thymus x citriodorus* [88]. In turn, **rosmarinic acid methylester (4)** was detected in distinct *Salvia* species [89,90] and in *T. vulgaris* [78].

More complex derivatives of rosmarinic acid like **lithospermic acid** (**5**), **methyl and ethyl derivatives of lithospermic acid (6-8)** [86,91,92], **prolithospermic** (**9**), **sagerinic acid (10)**, **salvinal (11)**, **magnesium lithospermate B (12)** and **ammonium-potassium lithospermate B (13)** are frequently found in *Salvia* genus [89], while they are rare in *Thymus*. Amongst those compounds, **lithospermic acid** is the most widely distributed one, as it was described to occur in *Salvia* species, mainly in *S. miltiorrhiza* [86,93] and in *T. serpyllum* [78] as well. In turn, the remaining compounds were mainly detected in

S. miltiorrhiza, which is the most studied *Salvia* species because of its vast usage in Chinese traditional medicine [86].

Notably, *Salvia* plants also typically contain other caffeic acid oligomers, which are overall named as **salvianolic acids**. Their name arises from the fact that they were isolated for the first time in *Salvia* genus. Chemically, these compounds are depsides (esters formed from two or more phenolic acids), in this particular case of 3, 4-dihydroxyphenyl-lactic acid and a caffeic acid derivative or a caffeic acid dimer, forming several types of carbon skeletons [94]. Among others, salvianolic acids **K, D, B** and **I** (**14-29**) have been largely described in *S. miltiorrhiza* [86,91–93,95–97]. Salvianolic acids were also found in other *Salvia* plants, including **salvianolic acid K (14)**, that is known to occur in *S. officinalis, S. fruticosa* and *S. pomifera*, with concentrations of 21.3, 15.3 and 6.7 mg/g dry plant, respectively [75], and **salvianolic acid E (23)**, was reported to occur in *Salvia veneris* [98] and in *Salvia reuterana* [99] **.**

Caffeic acid (**30**) i.e., the monomeric unit of rosmarinic acid and of its derivatives, was detected as a minor phenolic component in a high number of *Salvia* plants and in some *Thymus* species (Table 1). Reported levels of this hydroxycinnamic acid in *Salvia* and *Thymus* species are in the range of 0.1-2.96 and 0.1-5.17 mg/g of dry plant, respectively [66,67,73,80,85,100]. Simple caffeic acid derivatives also appear as minor phenolic constituents in the two plant genera herein in focus. **Caffeic acid hexoside (31)** was described to occur in *S. officinalis* [58] and in *T. vulgaris* [58,101], while **caffeic acid ethyl ester (32)** was identified in *T. serpyllum* [102]. Moreover, **chlorogenic acid (3-*O*-caffeoylquinic acid) (33)** was reported to occur in the methanolic extracts of different *Salvia* species: 0.06 and 0.09 mg/g dry plant in *S. argentea* [66] and in *S. cadmica* [71], respectively. Additionaly, chlorogenic acid was described to occur in several *Thymus* species, presenting contents in the range 0.02 and 7.88 mg/g of dry plant [80,81,83–85]. Other caffeic acid metabolites, known as **yunnaneic acids (34-41) A, B, C, D, E, F, G** and **H**, were identified in roots of *Salvia yunannesis* [103,104].

Besides caffeic acid and/or caffeic acid derivatives, several other hydroxycinnamic acids can often be found in the two plant genera. E.g. **ferulic acid (42)** was detected in the methanolic extracts of different *Salvia* species, with contents of 0.04-0.7 mg/g dry plant [66,71]. These coumpounds were described to occur in several *Thymus* species, whose amounts ranged from 0.03 to 4.6 mg/g of dry plant [80,84,105]. Moreover, **isoferulic acid (43)** is know to occur in *S. miltiorrhiza* [86] and *S. reuterana* [99].

Table 1 – Non-flavonoid phenolic compounds of Salvia and Thymus genera

Compound	*Salvia* species	*Thymus* species
Hydroxycinnamic acids		
Rosmarinic acid **(1)**	*S. amplexicaulis* [77,106], *S. euphratica* var. *euphratica, S. euphratica* var. *leiocalycina* [107], *S. cadmica* [71,77], *S. fruticosa* [1,72,108,109], *S. halophila* [76], *S. kronenburgii* [110], *S. miltiorrhiza* [86,92,93,97], *S. nemorosa* [73], *S. officinalis* [58,66–68,95,109,111–113], *S. pomifera* [109], *S. ringens* [114] *S. sclarea* [74], *S. splendens* [115], *S. syriaca* [116], *S. tomentosa* [70] *S. trichoclada, S. verticillata* [1,110], *S. veneris* [98] *S. aegyptiaca, S. argentea, S. verbenaca* [66], *S. aytachii, S. cassia, S. cerino-pruinosa, S. ekimiana, S. eriophora, S. freyniana, S. marashica, S. nutans, S. pilifera, S. potentillifolia, S. sericeo-tomentosa* var. *sericeo-tomentosa, S. sericeo-tomentosa* var. *hatayica, S. suffruticosa* x *bracteata, S. tchiatcheffii* [77], *S. azurea, S. forskaohlei, S. jurisicii, S. nemorosa, S. triloba* [117], *S. bowleyana, S. cavaleriei, S. chinensis, S. flava, S. lavandulifolia, S. prionitis, S. sonchifolia, S. yunnanensis* [118]	*T. capitatus* [84], *T. x citriodorus* [88], *T. lotocephalus* [119], *T. mastichina* [81,120],*T. nummularius* [83], *T. serpyllum* [78–80,85,102], *T. praecox ssp. grossheimii* [82], *T. pulegioides* [85,121], *T. quinquecostatus* var. *japonica* [122], *T. sipthorpii* [1], *T. sipyleus* [123] *T. glabrescens, T. pannonicus, T. praecox, T. pulegioides* [85] *T. vulgaris* [58,68,78,79,87,111–113,124,125]
Rosmarinic acid glucoside **(2)**	*S. miltiorrhiza* [86]	*T. serpyllum* [80], *T. vulgaris* [101]
(caffeoyl)rosmarinic acid **(3)**		*T. x citriodorus* [88], *T. vulgaris* [87]
Rosmarinic acid methylester **(4)**	*S. bowleyana, S. prionitis, S. sonchifolia* [118], *S. miltiorrhiza* [86] *S. plebeia* [90], *S. splendens* [126]	*T. vulgaris* [78]
Lithospermic acid **(5)**	*S. miltiorrhiza* [86,91–93], *S. reuterana* [99], *S. sonchifolia* [118]	*T. serpyllum* [78]
Lithospermic acid monomethyl ester **(6)**	*S. miltiorrhiza* [86]	
Lithospermic acid dimethyl ester **(7)**	*S. miltiorrhiza* [86]	
Ethyl lithospermate **(8)**	*S. miltiorrhiza* [86,91]	
Prolithospermic acid **(9)**	*S. miltiorrhiza* [89,91]	
Sagerinic acid **(10)**	*S. officinalis* [95]	
Salvinal **(11)**	*S. miltiorrhiza* [86]	
Magnesium lithospermate B **(12)**	*S. miltiorrhiza* [86]	
Ammonium-potassium lithospermate B **(13)**	*S. miltiorrhiza* [86]	

Salvianolic acid K **(14)**	*S. fruticosa, S. officinalis* [95,101,109], *S. pomifera* [109]	*T. vulgaris* [101]
Salvianolic acid D **(15)**	*S. miltiorrhiza* [92], *S. reuterana* [99]	
Salvianolic acid B **(16)**	*S. miltiorrhiza* [86,92,93,96], *S. reuterana* [99]	
Ethyl lithospermate B **(17)**	*S. miltiorrhiza* [86,91]	
Dimethyl lithospermate B **(18)**	*S. miltiorrhiza* [91]	
Salvianolic acid I **(19)**	*S. fruticosa, S. officinalis* [95,118], *S. pomífera* [109]	*T. vulgaris* [101]
Salvianolic acid A **(20)**	*S. miltiorrhiza* [86,92,93], *S. reuterana* [99]	
Salvianolic acid C **(21)**	*S. miltiorrhiza* [86,91,92]	
Isosalvianolic acid C **(22)**	*S. miltiorrhiza* [86]	
Salvianolic acid E **(23)**	*S. miltiorrhiza* [86,91,92], *S. reuterana* [99], *S. veneris* [98]	
Salvianolic acid H **(24)**	*S. officinalis, S. fruticosa, S. pomífera* [109]	
Salvianolic acid J **(25)**	*S. miltiorrhiza* [86,92]	
Salvianolic acid L **(26)**	*S. miltiorrhiza* [92]	
Norsalvianolic acid L **(27)**	*S. miltiorrhiza* [127]	
Salvianolic acid F **(28)**	*S. miltiorrhiza* [92,97,118], *S. officinalis, S. fruticosa, S. pomífera* [109]	
Salvianolic acid G **(29)**	*S. miltiorrhiza* [86,92,118]	
Caffeic acid **(30)**	*S. amplexicaulis* [106], *S. cadmica* [71,77], *S. bicolor* [128], *S. euphratica* var. *euphratica, S. euphratica* var. *leiocalycina* [107], *S. fruticosa* [1,72,108,109], *S. halophila* [76], *S. hispanica* [129], *S. miltiorrhiza* [86,92,93,97], *S. nemorosa* [73], *S. officinalis* [58,66–68,95,109,111–113,130,131], *S. pomifera* [109] *S. plebeia* [132], *S. reuterana* [99], *S. ringens* [114], *S. sclarea* [74], *S. splendens* [115], *S. syriaca* [116],*S. tomentosa* [70], *S. veneris* [98], *S. virgata* [133], *S. aegyptiaca, S. argentea, S. verbenaca* [66], *S. amplexicaulis, S. cadmica, S. cerino-pruinosa, S. nutans, S. pilifera, S. tchiatcheffii* [77], *S. azurea, S. forskaohlei, S. jurisicii, S. nemorosa, S. triloba* [117], *S. kronenburgii, S. trichoclada, S. vertisillata* subsp. *vertisillata* [110], *S. albimaculata, S. bowleyana, S. calycina, S. chinensis, S. horminum, S. limbata, S. plebeia, S. sonchifolia* [118]	*T. lotocephalus* [119], *T. quinquecostatus* [122] *T. serpyllum* [78,80,85], *T. sipthorpii* [1], *T. vulgaris* [68,112,113,130,134]
Caffeic acid hexoside **(31)**	*S. officinalis* [58], *S. veneris* [98]	*T. vulgaris* [58,101]
Caffeic acid ethyl ester **(32)**		*T. serpyllum* [102]

Chlorogenic acid **(33)**	*S. argentea* [66],*S. bicolor* [128], *S. cadmica* [71], *S. euphratica* var. *euphratica* [107], *S. fruticosa* [72,108], *S. nemorosa* [73], *S. officinalis* [58,131,135], *S. pilifera* [77], *S. tomentosa* [70], *S. sclareoides* [136], *S. syriaca* [116], *S. amplexicaulis, S. aytachii, S. ekimiana, S. freyniana, S. marashica, S. nutans, S. potentillifolia, S. sericeo-tomentosa* var. *sericeo-tomentosa, S. sericeo-tomentosa* var. *hatayica, S. suffruticosa* x *bracteata, S. tchiatcheffii* [77], *S. azurea, S. forskaohlei, S. jurisicii, S. nemorosa, S. triloba* [117] , *S. horminum, S. triloba* [118], *S. kronenburgii, S. trichoclada, S. vertisillata* subsp. *vertisillata* [110]	*T. capitatus* [84], *T. mastichina* [81], *T. nummularius* [83], *T. serpyllum* [80,102], *T. vulgaris* [58]
Yunnaneic acids A **(34)**, B **(35)**, C **(36)**, D **(37)**, E **(38)**, F **(39)**, G **(40)**, H **(41)**	*S. yunannesis* [103,104,137,138]	
Ferulic acid **(42)**	*S. bicolor* [139], *S. cadmica* [71], *S. euphratica* var. *euphratica, S. euphratica* var. *leiocalycina* [107], *S. fruticosa* [72,108] *S. miltiorrhiza* [92,97], *S. nemorosa* [73], *S. officinalis* [66,100,135,140] *S. reuterana* [99], *S. tomentosa* [70], *S. sclareoides* [136], *S. syriaca* [116], *S. aegyptiaca, S. argentea, S. verbenaca* [66], *S. amplexicaulis, S. aytachii, S. cadmica, S. cassia, S. cerino-pruinosa, S. ekimiana, S. eriophora, S. freyniana, S. marashica, S. nutans, S. pilifera, S. potentillifolia, S. sericeo-tomentosa* var. *hatayica, S. suffruticosa* x *bracteata, S. tchiatcheffii* [77]	*T. capitatus* [84], *T. hirtus* [105], *T. serpyllum* [80,85], *T. sipthorpii* [1], *T. vulgaris* [58,130] *T. glabrescens, T. pannonicus, T. pulegioides, T. praecox* [85]
Isoferulic acid **(43)**	*S. miltiorrhiza* [86], *S. reuterana* [99]	
p-coumaric acid **(44)**	*S. bicolor* [139], *S. cadmica* [71], *S. euphratica* var. *euphratica, S. euphratica* var. *leiocalycina* [107], *S. fruticosa* [72], *S. nemorosa* [73] *S. officinalis* [100,134,135], *S. reuterana* [99], *S. sclareoides* [136], *S. sericeo-tomentosa* var. *sericeo-tomentosa* [70], *S. syriaca* [116], *S. verbenaca* [66], *S. amplexicaulis, S. aytachii, S. cassia, S. cerino-pruinosa, S. ekimiana, S. eriophora, S. marashica, S. nutans, S. potentillifolia, S. suffruticosa* x *bracteata, S. tchiatcheffii* [77]	*T. capitatus* [84], *T. hirtus* [105], *T. serpyllum* [80,85], *T. sipthorpii* [1], *T. vulgaris* [58,130], *T. glabrescens, T. pannonicus, T. pulegioides, T. praecox* [85]
o-coumaric acid **(45)**	*S. bicolor* [128], *S. cadmica* [71,77], *S. euphratica* var. *euphratica, S. euphratica* var. *leiocalycina* [107], *S. halophila* [76], *S. nemorosa* [73,117], *S. syriaca* [116],*S. virgata* [133], *S. aytachii, S. cerino-pruinosa, S. ekimiana, S. freyniana, S. marashica, S. sericeo-tomentosa* var. *sericeo-tomentosa, S. sericeo-tomentosa* var. *hatayica* [77], *S. officinalis, S. triloba* [117]	
Sinapic acid **(46)**	*S. bicolor* [128], *S. cadmica* [71], *S. nemorosa* [73], *S. syriaca* [116]	

Hydroxybenzoic acids		
Gallic acid **(47)**	*S. argentea* [66], *S. bicolor* [128], *S. cadmica* [71], *S. euphratica* var. *euphratica, S. euphratica* var. *leiocalycina* [107], *S. fruticosa* [72], *S. halophila* [76], *S. officinalis* [58,66,117,135] *S. ringens* [114], *S. sclareoides* [136] *S. sericeo-tomentosa* var. *sericeo-tomentosa* [70]	*T. capitatus* [84], *T. hirtus* [105], *T. nummularius* [83] *T. serpyllum* [80], *T.sipthorpii* [1], *T. vulgaris* [58,68,141]
Gentisic acid **(48)**	*S. bicolor* [128], *S. officinalis* [134], *S. sclareoides* [136]	*T. vulgaris* [134]
Syringic acid **(49)**	*S. bicolor* [139], *S. cadmica* [71], *S. nemorosa* [73], *S. officinalis* [23,58,134], *S. sclareoides* [136], *S. syriaca,* [116] *S. amplexicaulis, S. cadmica, S. cassia, S. cerino-pruinosa, S. eriophora, S. freyniana, S. marashica, S. nutans, S. potentillifolia, S. sericeo-tomentosa* var. *sericeo-tomentosa, S. sericeo-tomentosa* var. *hatayica , S. tchiatcheffii* [77]	*T. capitatus* [84], *T. hirtus* [105], *T. serpyllum* [102], *T. sipthorpii* [1] *T. vulgaris* [58,134]
Protocatechuic acid **(50)**	*S. cadmica* [71], *S. euphratica* var. *euphratica* [107], *S. miltiorrhiza* [86,91,92,127], *S. nemorosa* [73], *S. officinalis* [58,117,131], *S. syriaca,* [116] *S. amplexicaulis, S. aytachii* , *S. bicolor, S. ekimiana, S. freyniana, S. potentillifolia* [77], *S. azurea, S. forskaohlei* [117]	*T. capitatus* [84], *T. nummularius* [83], *T. quinquecostatus* [122] *T. serpyllum* [80], *T. vulgaris* [58]
Protocatechuic ethyl ester **(51)**	*S. hispanica* [142]	
Protocatechuic aldehyde **(52)**	*S. miltiorrhiza* [86,91–93,127]	
p- hydroxybenzoic acid **(53)**	*S. bicolor* [139], *S. fruticosa* [108], *S. halophila* [76], *S. nemorosa* [73] *S. miltiorrhiza* [127], *S. officinalis* [58], *S. syriaca,* [116], *S. virgata* [133] *S. argéntea, S. verbenaca* [66], *S. amplexicaulis, S. ekimiana, S. aytachii, S. cadmica, S. cassia, S. nutans, S. pilifera, S. potentillifolia* [77]	*T. capitatus* [84], *T. serpyllum* [102], *T. vulgaris* [58]
Vanillic acid **(54)**	*S. fruticosa* [72,108], *S. officinalis* [58,112,135], *S. sclareoides* [136], *S. sericeo-tomentosa* var. *sericeo-tomentosa* [70], *S. verbenaca* [66], *S. aytachii, S. cadmica, S. cassia, S. ekimiana, S. eriophora, S. freyniana, S. marashica, S. pilifera, S. potentillifolia, S. sericeo-tomentosa* var. *sericeo-tomentosa, S. sericeo-tomentosa* var. *hatayica, S. suffruticosa* x *bracteata, S. tchiatcheffii* [77]	*T. capitatus* [84], *T. serpyllum* [102], *T. vulgaris* [58]
Hydroxyphenylpropanoic acids		
Danshensu **(55)**	*S. miltiorrhiza* [86,91,92,97,118,127], *S. officinalis* [131], *S. chinensis, S. prionitis, S. sonchifolia* [118]	
Danshensu methyl ester **(56)**	*S. miltiorrhiza* [127]	

Another hydroxycinnamic acid, ***p*-coumaric acid (44)**, was described to be present in different *Salvia* plants, with contents varying between 0.02-0.14 mg/g dry plant [66,71]. This compound was also found in different *Thymus* plants, namely *T. capitatus* (concentration of 0.1 mg/g dry plant) [84]. In addition, its structural isomer (***o*-coumaric acid (45)**) was largely described in *Salvia* plants [71,73,116,128]. Moreover, **sinapic acid (46)** was reported in different *Salvia* species [71,73,116] and curiously, its amounts in *Salvia bicolor* (1.53 mg/g dry plant) reached 8% of the total quantified phenolics [128].

1.2.1.2. Hydroxybenzoic acids

Hydroxybenzoic acids commonly appear as minor phenolic components in *Salvia* and *Thymus* plants (Table 1). In more detail, **gallic acid (47)** was found in many species, with contents in *S. officinalis* and *S. argentea* reported to amount for 0.03 and 0.02 mg/g of dry plant, respectively [66], while *S. bicolor* presented 4.1 mg/g of dry plant [128]. Furthermore, its content in *Thymus hirtus* sp. *algeriensis* and wild thyme (*T. serpyllum*) accounted for 2.8 and 0.6 mg/g of dry plant, respectively [80,105]. In addition, **gentisic acid (48)** was detected in *S. bicolor* [128] and in *T. vulgaris* [143], while **syringic acid (49)** was found in several *Salvia* [71,73,116] and *Thymus* [84,102,105] species. Other hydroxybenzoic acids in sage and thyme plants include **protocatechuic acid (50)** [71,73,80,83,84,116,131]. Additionaly, derivatives of this compound, particularly **protocatechuic ethyl ester (51)** was reported in *Salvia hispanica* [142], and **protocatechuic aldehyde (52)**, was described to be a phenolic constituent of *Thymus quinquecostatus* var. *japonica* [122], *S. miltiorrhiza* [86,92,93] and *S. officinalis* [67]. Likewise, ***p*-hydroxybenzoic acid (53)** and **vanillic acid (54)** were exhaustively reported in different species of *Salvia* plants [73,108,116] (see Table 1). Moreover, **vanillic acid** was described to occur in several *Thymus* species, with amounts estimated between 0.06 and 1.2 mg/g dry plant [84,102,105].

1.2.1.3. Others

Notably, other non-flavonoid phenolic constituents were previously detected in *Salvia* plants. E.g. **danshensu (55)**, a hydroxyphenylpropanoic acid, is known to occur in high amounts in *S. officinalis* and in *S. miltiorrhiza* [67,86,92,93,97,131,137], although it was also identified in other species, like *S. veneris* [98]. In addition, a methyl derivative of this compound **(56)** was detected in *S. miltiorrhiza* [127].

1 $R_1 = R_2 = H$
2 $R_1 =$ Glc $R_2 = H$
4 $R_1 = H$ $R_2 = CH_3$

3

5 $R_1 = R_2 = H$
6 $R_1 = H$ $R_2 = CH_3$
7 $R_1 = R_2 = CH_3$
8 $R_1 = C_2H_5$ $R_2 = H$

9

10

11

12 R= Mg^{2+}
13 R= $NH_4^+ + K^+$

14

Fig. 5: (*Contd*...)

15

16 R_1= H R_2= H
17 R_1= H R_2= C_2H_5
18 R_1= CH_3 R_2= CH_3

19

20 R= X
28 R= H

21

22

23

24

25

26

27

Fig. 5: (*Contd...*)

29

30 R_1= R_2= H
31 R_1= Hex R_2= H
32 R_1= H R_2= CH_2CH_3

33 R_3= R_2=H R_1= Caf

34 R= H
35 R= OH

36

37

Fig. 5: (*Contd...*)

42 R_1= OH R_2= OCH_3 R_3= R_4= H
43 R_1= OCH_3 R_2= OH R_3= R_4= H
44 R_1= OH R_2= R_3= R_4= H
45 R_1= R_2= R_4= H R_3= OH
46 R_1= OH R_2= R_4= OCH_3 R_3= H

47 R_1= R_2= R_3= OH R_4= R_5= H
48 R_1= R_4= OH R_2= R_3= R_5= H
49 R_1= R_3= OCH_3 R_2= OH R_4= R_5= H
50 R_1= R_4= R_5= H R_2= R_3= OH
51 R_1= R_4= H R_2= R_3= OH R_5= CH_2CH_3
53 R_1= R_3= R_4= R_5= H R_2= OH
54 R_1= R_4= R_5= H R_2= OH R_3= OCH_3

55 R= H
56 R= CH_3

Caff- Caffeoyl unit; Glc- Glucosyl unit; Hex- Hexosyl unit

Figure 5 – Chemical structures non-flavonoid phenolics reported in *Thymus* and *Salvia* plants.

1.2.2. Flavonoids

Flavonoids are characterized by a C15 structure (C6-C3-C6) with a heterocyclic benzopyran ring (C ring), an aromatic ring (A ring) and a phenyl constituent as the B ring (Figure 6A), all of them with several structural variations. They are divided into six main subclasses: flavonols, flavones, flavanones, flavan-3-ols, isoflavones and anthocyanidins, according to their oxidation state, the connection of an aromatic ring and the functional groups of the C ring. In the physiological state, flavonoids occur usually in association with sugar as glycosides [59,60,62–64,144].

In the particular case of *Salvia* and *Thymus* plants, special emphasis must be devoted to flavones, flavonols and flavanones. Their occurrence in the two plant genera are resumed in Tables 2, 3 and 4 and the corresponding structures are depicted in Figures 7, 8 and 9, respectively.

The sub-classe of flavones are characterized by the presence of a double bond between 2 and 3 position, containing several A- and B-rings substitutions and lacking oxygenation at the 3-position of the C-ring (Figures 6B), while flavonols and flavanones are respectively characterized by a 3-hydroxyflavone backbone and absence of the double bond between the 2- and 3-positions and the presence of a chiral center at the 2-position of the C-ring (Fig. 6C-D).

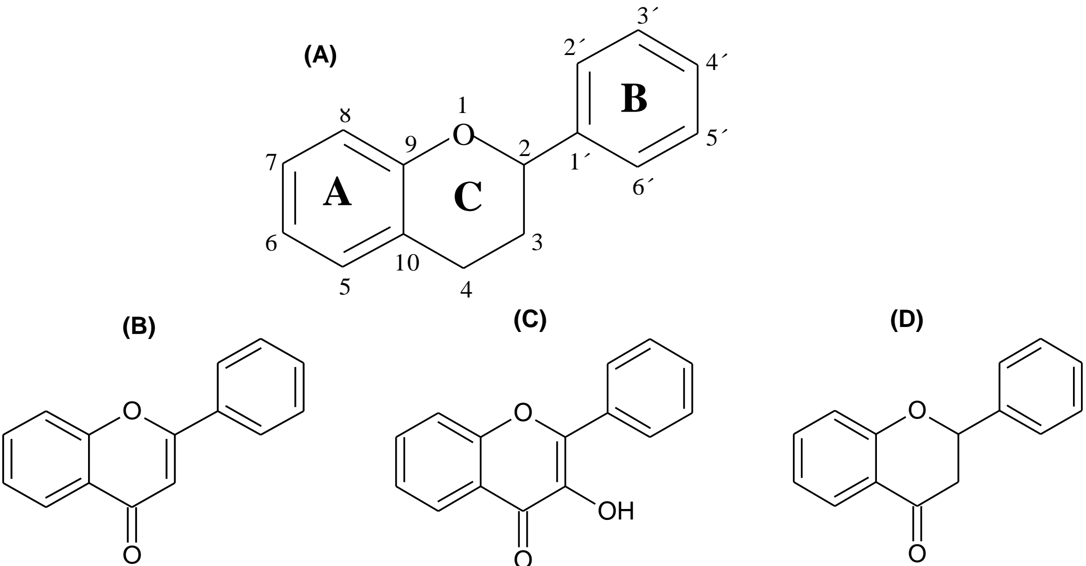


Figure 6 –General structure of flavonoids (A); flavones (B); flavonols (C); flavanones (D)

1.2.2.1. Flavones

Amongst flavones, sage and thyme plants mainly contain mono-glycosidic derivatives of luteolin and apigenin (Table 2 and Figure 7). Luteolin-*O*-glucosides (with prevalence of **luteolin-7-*O*-glucoside (57)**) are the most common *O*-glycosidic derivatives in *Salvia*

and *Thymus* species. In fact, these were previously detected in *S. officinalis* [66,131,145,146] as well as in other *Salvia* species, including *S. sclarea, S. argentea, S. aegyptica* and *S. fruticosa*, with amounts of 0.08 – 0.9 mg/g of dry plant [66,74,108]. **Luteolin-7-*O*-glucoside** was also reported in *Thymus*, namely in a hydromethanolic extract of *T. serpyllum* (accounting for 51.8 mg/g dry plant) [80], as well in other distinct *Thymus* species [88,124,141,147]. Luteolin-*O*-glucosides derivatives detected in *Thymus* plants also include **luteolin-7-*O*-(6"-feruloyl)-ß-glucopyranoside (58)**, which was found in *Thymus sipyleus* [123] and *Thymus schimperi* [147].

Other sugars such as glucuronic acid and rutinose are also frequently linked to the flavone skeleton on *Salvia* and *Thymus* plants. The **7-*O*-glucuronide derivative of luteolin (59)** was previously detected in *S. officinalis* [101,131,148,149] and in *T. serpyllum* (9 and 14 mg/g of dry plant) [78,80], among other *Thymus* species [78,88,123,147]. In turn, **luteolin-3'-*O*-glucuronide (60)** together **with luteolin-*O*-diglucuronide (61)** were found in *S. officinalis* [131,145,149], with the latter also occurring in *S. fruticosa*, *S. pomifera* [75] and in *T. serpyllum* [80]. *O*-rutinoside derivatives of luteolin (or in particular **7-*O*-rutinoside (62)**) were detected in sage plants such as *S. officinalis*, *S. fruticosa* and *S. pomifera* [67,75], as well as in *Thymus* plants [58,78], namely in *T. vulgaris* and *T. serpyllum,* where it was reported to account for 1.4 mg/g of dry plant [78]. Less frequent luteolin derivatives on the two plant genera comprise **6-hydroxyluteolin-7-*O*-glucoside (63)** and **6-hydroxyluteolin-7-*O*-glucuronide (64)**, which occur in *S. officinalis* [75,149] and the **luteolin-3'-methylether (65)**, which was previously reported in *Salvia palaestina* [150].

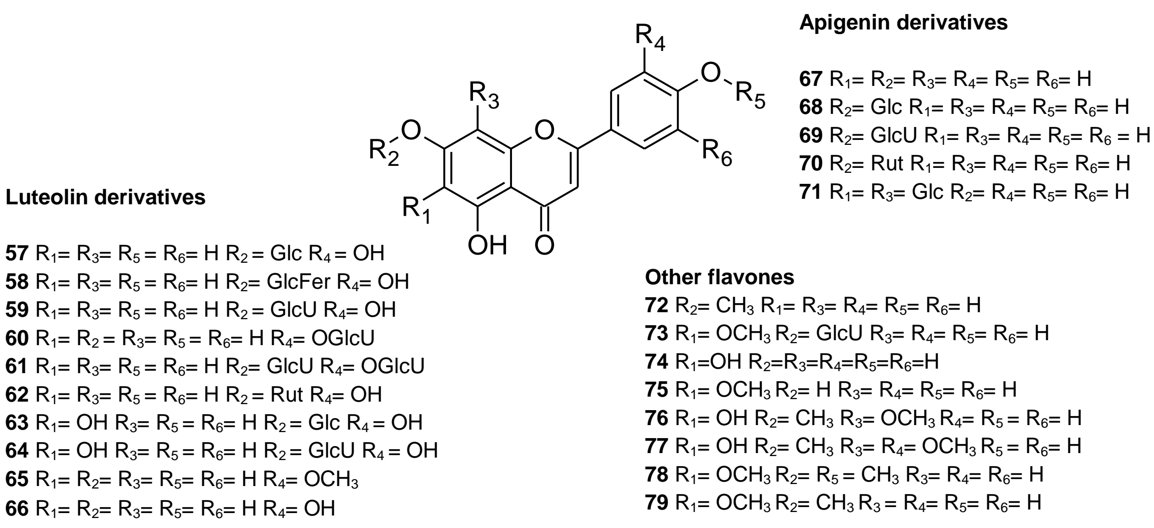
In turn, **luteolin (66)** (i.e. the aglycone form of the above compounds) is widely reported in *Salvia* and *Thymus* (Table 2). This was found as a major phenolic compound in *T. serpyllum* (48.04 mg/g dry plant) [80,102], although in the majority of cases, it represents a minor phenolic component. Concentrations in different populations of *T. mastichina* were reported in the range of 0-0.7 mg/g dry plant [81], while levels in other *Thymus* species varied between 0.36 and 0.55 mg/g of dry plant [69,78,84,134]. Moreover, levels of 0.26 to 0.50 mg/g dry plant were previously described for *S. officinalis* and *S. cadmica*, respectively [71,100].

In addition to luteolin and/or luteolin derivatives, considerable amounts of apigenin and/or apigenin glycosides are frequently reported in sage and thyme plants. As shown in Table 2, **apigenin (67)** contents in *Salvia* and *Thymus* species were found to vary in the ranges 0.006 - 0.22 mg/g and 0.04 – 0.12 mg/g dry plant, respectively [66,71,100].

Apigenin-7-*O*-glucoside (68) is the most typical *O*-glycosidic form of apigenin found in the two plant genera. This has been reported in several *Salvia* species, namely *S. officinalis*, *S. sclarea* and *S. fruticosa* [74,75,108,131], with a content of 1.1 mg/g plant reported for *S. aegyptaca* [66]. This flavone was also described to be a phenolic constituent of several *Thymus* species [58,79,85,113,124], with a content of 0.028 mg/g dry plant from *T. glabrescens* [85].

Others glycosidic derivatives of apigenin previously described to be present in *Salvia* and/or *Thymus* species comprise **apigenin-7-*O*-glucuronide (69)**, which was detected in *T. vulgaris, T. serpyllum, T. x citriodorus* and *S. officinalis* [67,80,88,101], and **apigenin-7-*O*-rutinoside (70)**, known to occur in *Salvia* plants [58,75]. Albeit less frequent, the **6,8-di-*C*-glucoside** derivative of apigenin **(71)** was already reported in *S. officinalis* [95,149].

Other flavones besides the luteolin and apigenin derivatives can exist in *Salvia* and *Thymus* plants, though with much less frequency. E.g. **genkwanin aglycone (72)** was detected in *S. officinalis* [66] and in different thyme species [151], while **hispidulin-*O*-glucuronide (73)** was found in sage species [75,101]. In turn, **scutellarein (74)** was described in *S. officinalis* [112], and **hispidulin (75)** was identified in *Salvia plebeia, S. officinalis* and in *T. vulgaris* [112,132]. Other rare aglycones derived from methylated flavones might also occur in thyme and sage plants, namely **thymusin (76)** and **thymonin (77)**, which was described in several *Thymus* species [151], together with **salvigenin (78)**, known to occur in *S. apiana* [152] and in *Salvia chloroleuca* [153], while **cirsimaritin (79)**, was also detected in *S. apiana* [152], in *T. serpyllum* [102] and in other *Thymus* species [151].



Fer- Feruloyl unit;Glc- Glucosyl unit; GlcU- Glucuronyl unit; Rut- Rutinosyl unit

Figure 7 – Chemical structures of flavones reported in *Salvia* and *Thymus* plants

Table 2 – Flavones of *Salvia* and *Thymus* genera

Compound	*Salvia* species	*Thymus* species
Luteolin-*O*-glucoside **(57)**	*S. aegyptiaca, S. argentea* [66] *S. fruticosa* [108,109], *S. officinalis* [66,67,109,113,131,141] *S. plebeia* [132], *S. sclarea* [74], *S. veneris* [98]	*T. x citriodorus* [88], *T. serpyllum* [79,80], *T. schimperi* [147], *T. sipyleus* [123], *T. vulgaris* [79,113,124,141], *T. zygis* [124]
Luteolin-7-*O*-(6”-feruloyl)-ß-glucopyranoside **(58)**		*T. schimperi* [147], *T. sipyleus* [123]
Luteolin-7-*O*-glucuronide **(59)**	*S. fruticosa* [109], *S. officinalis* [67,109,131] *S. pomifera* [109], *S. palaestina* [150] *S. veneris* [98]	*T. x citriodorus* [154], *T. schimperi* [147] *T. serpyllum* [78,80], *T. sipyleus* [123], *T. vulgaris* [78,112]
Luteolin-3’-*O*-glucuronide **(60)**	*S. officinalis* [95,149]	
Luteolin-*O*-diglucuronide **(61)**	*S. officinalis* [109,131], *S. fruticosa, S. pomifera* [109]	*T. serpyllum* [80]
Luteolin-*O*-rutinoside **(62)**	*S. officinalis* [67,109,112], *S. fruticosa, S. pomifera* [109]	*T. serpyllum* [78], *T. vulgaris* [58,78,112]
6-hydroxyluteolin-7-*O*-glucoside **(63)**	*S. officinalis* [109,149], *S. fruticosa, S. pomifera* [109]	
6-hydroxyluteolin-7-*O*-glucuronide **(64)**	*S. officinalis* [109,149], *S. fruticosa, S. pomifera* [109]	
Luteolin-3’-methylether **(65)**	*S. palaestina* [150]	
Luteolin **(66)**	*S. aegyptiaca* [66],*S. amplexicaulis*[106], *S. argentea* [66], *S. cadmica* [71], *S. chloroleuca* [153], *S. fruticosa* [72,108,109], *S. nemorosa* [73] *S. officinalis* [66,67,100,109,112], *S. plebeia* [90,132], *S. ringens* [114], *S. sclarea* [74], *S. syriaca* [116], *S. tomentosa* [70], *S. verbenaca* [66]	*T. capitatus* [84], *T. lotocephalus* [119] *T. mastichina* [81,120], *T. nummularius* [83] *T. schimperi* [147], *T. serpyllum* [78,80,102] *T. vulgaris* [78,112,134]
Apigenin **(67)**	*S. argentea, S. aegyptiaca* [66], *S. amplexicaulis* [106], *S. cadmica* [71], *S. fruticosa* [72,108,109], *S. nemorosa*[73], *S. officinalis* [66,67,100,109,112,113], *S. pomifera* [109], *S. ringens* [114], *S. sclarea* [74], *S. syriaca* [116], *S. tomentosa* [70], *S. verbenaca* [66]	*T. lotocephalus* [119], *T. mastichina* [120], *T. nummularius* [83], *T. serpyllum, T. glabrescens, T. pannonicus,T. praecox, T. pulegioides* [85], *T. vulgaris* [78,100,113]
Apigenin-7-*O*-glucoside **(68)**	*S. argentea, S. aegyptiaca* [66], *S. fruticosa* [108,109], *S. officinalis* [66,109,113,131], *S. pomifera* [109], *S. sclarea* [74]	*T. x citriodorus* [154] *T. glabrescens,T. pannonicus, T. praecox, T. pulegioides* [85], *T. serpyllum* [79,85] *T. vulgaris* [58,79,113,124,141]
Apigenin-7-*O*-glucuronide **(69)**	*S. officinalis* [67,109,131], *S. fruticosa, S. pomifera* [109]	*T. x citriodorus* [154], *T. serpyllum* [80], *T. vulgaris* [101]
Apigenin-7-*O*-rutinoside **(70)**	*S. officinalis* [58,109], *S. fruticosa, S. pomifera* [109]	*T. vulgaris* [58]
Apigenin-6,8-di-*C*-glucoside **(71)**	*S. officinalis* [95,149]	
Genkwanin **(72)**	*S. aegyptiaca, S. officinalis, S. verbenaca* [66]	*T. comptus, T. glabrescens, T. longidens, T. pulegioides, T. tosevii, T. thracicus* [151]
Hispidulin-glucuronide **(73)**	*S. officinalis* [101,109], *S. fruticosa, S. pomifera* [109]	
Scuttelarein **(74)**	*S. officinalis* [112]	
Hispidulin **(75)**	*S. officinalis* [112], *S. plebeian* [132]	*T. vulgaris* [112]
Thymusin **(76)**		*T. alsarensis, T. glabrescens, T. longidens, T. macedonicus, T. oehmianus, T. thracicus, T. tosevii* [151]
Thymonin **(77)**		*T. alsarensis, T. glabrescens, T. macedonicus, T. oehmianus, T. tosevii, T. thracicus,* [151]
Salvigenin **(78)**	*S. apiana* [152], *S. chloroleuca* [153]	*T. albanus, T. grisebachii, T. longidens, T. pseudoatticus* [151]
Cirsimaritin **(79)**	*S. apiana* [152]	*T. grisebachii, T. pseudoatticus* [151], *T. serpyllum* [102]

1.2.2.2. Flavonols

The most common flanonols in *Salvia* and *Thymus* plants include quercetin and kaempferol, as well as their derivatives (Table 3 and Figure 10). **Quercetin (80)** was detected in several *Salvia* species [1,71,114], namely in the methanolic extract of *S. nemorosa* (0.02 mg/g dry plant) [73] and in *S. officinalis* and *S. fruticosa,* with contents of 1.8 and 0.8 mg/g of dry plant, respectively [72,100,108]. In *Thymus*, this flavonol was previously detected in diverse species [83,85,120] and levels of 0.023 mg/g dry plant were reported in *T. capitatus* [84].

Quercetin-3-*O*-rutinoside i.e., rutin (81) is the most common quercetin derivative of the two plant genera. This was already found in several *Salvia* species, including *Salvia ringens, Salvia amplexicaulis, Salvia syriaca, S. miltiorrhiza* and *S. nemorosa* [73,93,106,114,116]. Likewise, its occurrence was described in *Thymus* plants, such as *T. capitatus, T. hirtus* and *T. serpyllum* (0.008, 0.6 and 1.35 mg/g dry plant, respectively) [80,84,105]. Other quercetin derivatives, namely **quercetin-3-*O*-glucoside (82)** and **dihydroquercetin (83)** were detected in *S. fruticosa* with contents of 0.28 and 0.016 mg/g dry plant, respectively [108], having the latter compound also been reported in different *Thymus* species, including *T. glabrescens, T. pannonicus, T. serpyllum* and *T. pulegioides* [85]. Moreover, quercetin-**3-*O*-galactoside (84)** was detected in *S. officinalis* and in *T. vulgaris* [113]. On the other hand, **kaempferol (85)** was described as a phenolic constituent of *S. fruticosa* and *S. cadmica* (0.054 and 0.056 mg/g dry plant, respectively) [71,108] among others [73,155]. Its occurrence was also previously described in *Thymus* plants, namely *T. capitatus* (0.049 mg/g dry plant) [84] and *T. nummularis* (0.12 mg/g dry plant) [83]. Additionally, **kaempferol-3-glucoside (86)** and **dihydrokaempferol (87)** was detected in *S. fruticosa* [108]. In turn, **kaempferol-*O*-rhamnoside (88)** together with **kaempferol-*O*-3-glucoside** have only been found in *Salvia* plants [106,108,114], while **kaempferol-*O*-glucoronide (89)** and **methyl-kaempferol-*O*-rutinoside (90)** was reported for *T. serpyllum* (15.2 and 17.4 mg/g dry plant, respectively) [80]. Moreover, **isorhamnetin (91)** and **isorhamnetin-*O*-hexoside (92)** are known to be present in *Thymus* plants (*T. vulgaris* [58]), while **morin (93)** and/or **myricetin (94)** was identified in sage plants, namely *S. fruticosa, S. tomentosa* and *S. hispanica* [70,72,129].

HO O OH R^1 OH OH O

83 R_1=OH
87

Quercetin derivatives

80 R_1= R_2= R_4= R_5= R_6= H R_3= OH
81 R_1= R_2= R_4= R_5= H R_3= OH R_6= Rut
82 R_3= OH R_1= R_2= R_4= R_5= H R_6= Glc
84 R_3= OH R_1= R_2= R_4= R_4= H R_6= Gal

Kaempferol derivatives

85 R_1= R_2= R_3= R_4= R_5= R_6= H
86 R_1= R_2= R_3= R_4= R_5= H R_6= Glc
88 R_1= R_2= R_3= R_4= R_5= H R_6= Rha
89 R_1= R_2= R_3= R_4= R_5= H R_6= GlcU
90 R_1= R_2= R_3= R_4= R_5= H R_6= Rut

Other flavonols

91 R_3= OCH_3 R_1= R_2= R_4= R_5= R_6= H
92 R_1= R_2= R_4= R_5= H R_3= OCH_3 R_6= Hex
93 R_1= R_3= R_4= R_5= R_6= H R_2= OH
94 R_1= R_2= R_4= R_6= H R_3= R_5= OH

Gal- Galactosyl unit; Glc- Glucosyl unit; GlcU- Glucuronyl unit; Hex - Hexosyl; Rha- Rhamnosyl unit; Rut- Rutinosyl unit

Figure 8 - Chemical structures of flavonols reported in *Salvia* and *Thymus* plants

Table 3 – Flavonols of *Salvia* and *Thymus* genera.

Compound	*Salvia* species	*Thymus* species
Quercetin **(80)**	*S. cadmica* [71], *S. fruticosa* [72,108], *S. hispanica* [129], *S. nemorosa* [73], *S. officinalis* [100,135], *S. ringens* [114], *S. syriaca* [116], *S. tomentosa* [70]	*T. capitatus* [84], *T. mastichina* [120], *T. nummularius* [83], *T. glabrescens, T. pannonicus, T. praecox, T. pulegioides* [85], *T. serpyllum* [79,85], *T. vulgaris* [58,79,130]
Rutin **(81)**	*S. amplexicaulis* [106], *S. fruticosa* [72], *S. miltiorrhiza* [93], *S. nemorosa* [73], *S. officinalis* [113], *S. syriaca* [116], *S. ringens* [114], *S. tomentosa* [70]	*T. capitatus* [84], *T. hirtus* [105], *T. nummularius* [83], *T. glabrescens, T. pannonicus, T. praecox, T. pulegioides* [85], *T. serpyllum* [80,85], *T. vulgaris* [58,113]
Quercetin-3-*O*-glucoside **(82)**	*S. fruticosa* [108], *S. officinalis* [58]	
Dihydroquercetin (83)	*S. fruticosa* [108]	*T. glabrescens, T. pannonicus, T. praecox, T. pulegioides, T. serpyllum* [85]
Quercetin-3-*O*-galactoside **(84)**	*S. officinalis* [113,135]	*T. vulgaris* [113]
Kaempferol **(85)**	*S. cadmica* [71], *S. fruticosa* [72,108] *S. hispanica* [129], *S. nemorosa* [73] *S. officinalis* [135,155], *S. syriaca* [116], *S. tomentosa* [70]	*T. capitatus* [84], *T. mastichina* [120] *T. nummularius* [83]
Kaempferol-3-glucoside **(86)**	*S. fruticosa* [108]	
Dihydrokaempferol **(87)**	*S. fruticosa* [108]	
Kaempferol-rahmnoside **(88)**	*S. amplexicaulis* [106], *S. ringens* [114]	
Kaempferol-*O*-glucuronide **(89)**		*T.serpyllum* [80]
Kaempferol-*O*-rutinoside **(90)**		*T.serpyllum* [80]
Isorhamnetin **(91)**	*S. officinalis* [58]	*T. vulgaris* [58]
Isorhamnetin-hexoside **(92)**	*S. officinalis* [58], *S. veneris* [98]	*T. vulgaris* [58]
Morin **(93)**	*S. fruticosa* [72], *S. tomentosa* [70]	
Myricetin **(94)**	*S. fruticosa* [72], *S. hispanica* [129], *S. tomentosa* [70]	

1.2.2.3. Flavanones

Amongst flavanones, naringenin, eriodictyol and hesperitin, along with some of their glycosidic forms are known to exist in *Thymus* and *Salvia* plants (Table 4 and Fig. 5). **Naringenin (95)** was detected in *S. mexicana, S. fruticosa, S. argentea* and *S. verbenaca* [66,108,156] and in distint *Thymus* species such as *T. capitatus* (8.3 mg/g of dry plant) [84] and *T. nummularis* (0.064 mg/g dry plant) [83]. In addition, the content of this polyphenolic compounds on dry-weight basis in *T. pulegioides* and in *T. pannonicus* was about 0.025 mg/g dry plant [85]. Instead, the **7-*O*-rutinoside** derivative of naringenin **(96)** was identified in *T. vulgaris* and in *S. officinalis*, accounting for 0.3 and 0.04 mg/g of dry plant, respectively [78,135]. Other naringenin-*O*-derivative, the **naringenin-*O*-glucoside (97)**, was also reported in *T. vulgaris* and *T. x citriodorus* species (0.3 and 0.6 mg/g of dry plants, respectively) [78,88].

Eriodictyol and/or eriodictyol derivatives are typiccaly found in *Salvia* and *Thymus* plants (Table 4 and Figure 5). Among them, **eriodictyol (98)** was previoulsy detected in *S. syriaca, S. cadmica* and *S. nemorosa* [71,73,116] and in several *Thymus* species [41,85,101,147,151], namely *T. vulgaris* (1.5 mg/g of dry plant) [78]. Moreover, hexosides of this flavanone, namely **eriodictyol-*O*-glucoside (99)** and **eriodictyol-*O*-glucuronide (100)**, are known to occur in *T. vulgaris* [78,101] and **eriocitrin** (eriodictyol-7-*O*-rutinoside) **(101)** was reported in *S. officinalis* [131]. Some of these derivatives are also known to occur in *T. x citriodorus* [88] and/or in *T. serpyllum* [102].

Beyond the most prevalent flavanones, **hesperidin** (hesperetin-7-*O*-rutinoside) **(102)** was described to account for 1 mg/g of dry plant in *T. vulgaris* [78] and in *Salvia* species [73,108,116], such as *S. cadmica* and *S. verbenaca* (0.2 and 0.46 mg/g of dry plant, respectively) [66,71]. Furthermore, **hesperetin (103)** was identified in *S. fruticosa* [72], in *S. tomentosa* (1.0 mg/g dry plant) [70] and in *T. pulegioides, T. serpyllum* and *T. praecox* [85].

Flavanones

95 R_1= R_2= H R_3= OH
96 R_1= Rut R_2= H R_3= OH
97 R_1= Glc R_2= H R_3= OH
98 R_1= H R_2= R_3= OH
99 R_1= Glc R_2= R_3= OH
100 R_1= GlcU R_2= R_3= OH
101 R_1= Rut R_2= R_3= OH
102 R_1= Rut R_2= OH R_3= OCH_3
103 R_1= H R_2= OH R_3= OCH_3

Glc- Glucosyl unit; GlcU- Glucuronyl unit; Rut- Rutinosyl unit

Figure 9 – Chemical structures of flavanones reported in *Salvia* and *Thymus* plants.

Table 4 – Flavanones of *Salvia* and *Thymus* genera

Flavanones	*Salvia* species	*Thymus* species
Naringenin **(95)**	*S. argentea, S. verbenaca* [66] *S. fruticosa* [108], *S. mexicana* [156]	*T. capitatus* [84], *T. glabrescens* [85,151], *T. nummularius* [83], *T. pannonicus, T. praecox* [85], *T. pulegioides* [85,151] *T. serpyllum* [41,85], *T. vulgaris* [78] *T. longicaulis, T. rohlenae, T. moesiacus, T. jankae, T. albanus, T. balcanus, T. oehmianus, T. tosevii, T. grisebachii, T. longidens, T. thracicus, T. pseudoatticu* [151],
Naringenin-7-*O*-rutinoside **(96)**	*S. officinalis* [135]	*T. vulgaris* [78]
Naringenin-*O*-glucoside **(97)**		*T. vulgaris* [78]
Eriodictyol **(98)**	*S. cadmica* [71], *S. nemorosa* [73], *S. syriaca* [116]	*T. glabrescens* [85,151], *T. longicaulis, T. rohlenae, T. moesiacus, T. jankae, T. albanus, T. balcanus, T. oehmianus*, *T. tosevii, T. grisebachii, T. longidens, T. thracicus, T. pseudoatticu* [151], *T. pannonicus, T. praecox* [85], *T. pulegioides* [85,151], *T. serpyllum* [41,85] *T. schimperi* [147], *T. vulgaris* [78,87,101]
Eridioctyol-*O*-glucoside (**99)**		*T. x citriodorus* [88], *T. vulgaris* [78,101]
Eridioctyol-*O*-glucuronide **(100)**		*T. serpyllum* [102], *T. vulgaris* [101]
Eriocitrin **(101)** (eriodictyol-7-O-rutinoside)	*S. officinalis* [131]	*T. serpyllum* [78,79], *T. vulgaris* [78,79]
Hesperidin **(102)** (hesperetin-7-O-rutinoside)	*S. cadmica* [71], *S. fruticosa* [108], *S. nemorosa* [73], *S. syriaca* [116], *S. verbenaca* [66]	*T. nummularius* [83], *T. vulgaris* [78]
Hesperetin **(103)**	*S. fruticosa* [72], *S. tomentosa* [70]	*T. pannonicus* [85], *T. praecox* [85] *T. pulegioides* [85], *T. serpyllum* [85]

1.3. Beneficial effects

Several plants belonging to *Salvia* and *Thymus* genera have been recognized to have potential uses in medical procedures and applications in pharmaceutical and cosmetic industries because their biological properties [1,14,157]. Several researchers found that polar extracts are rich in phenolic compounds, particularly phenolic acids and flavonoids, which are related with their biological properties, namely antimicrobial, antioxidant, anti-inflammatory, among others [19,20,47]. These health beneficial activities can combat oxidative stress conditions, protecting against chronic diseases such as cardiovascular diseases, diabetes, cancers, ageing and other degenerative diseases in humans [1,158,159]. A summary of the main beneficial properties associated to the plant genera target in this work is given below, highlighted the most relevant studies (Tables 5, 6, 7 and 8). Particular emphasis will be devoted to antioxidant, anti-inflammatory, anticancer and antibacterial properties, since these are the most exploited ones up to the moment for *Salvia* and *Thymus* genus, as well as those investigated in the practical part of the present work.

1.3.1. Antioxidant activity

Oxidative stress is a condition characterized by an imbalance between pro-oxidants and antioxidant defenses. Reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), together with reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), are generated in a variety of intracellular processes. To minimize the oxidative damage, enzymatic and non-enzymatic antioxidants have been evolved. Enzymatic antioxidants include superoxide dismutase (SOD), which catalyze $ONOO^-$ to produce H_2O_2, that in its turn, can be neutralized by catalase or glutathione peroxidase. In turn, non-enzymatic antioxidants also counteract oxidative damage, among them, vitamins A, C and E, glutathione (reduced form, GSH), and β-carotene provide major protection against oxidative stress [160–163].

In normal physiological conditions, cells are able to maintain a redox homeostasis, i.e., the balance between reactive species formation and their elimination. Still, when equilibrium is disrupted, oxidative stress is settled, and in this biological condition, the overproduction of reactive species leads to several mitochondrial and cellular damages, namely in lipids, proteins, DNA and other macromolecules, that is closely associated to aging processes [164,165] and to the physiopathology of several inflammatory-related diseases [160,163,166], including initiation and/or development of cancer [163,167,168].

Diet supplementation with antioxidant compounds by a daily consumption of plant-based products, that contains large amount of polyphenols and phytochemicals, could provide an important tool to counteract the adverse effects of oxidative stress [2,20,160]. Its antioxidant properties are involved in the prevention of several diseases [1,2,169], indicating an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human disease [160,170].

Many plants, including Lamiaceae species, have been used in traditional medicine for their claimed antioxidant capacities and/or ability to ameliorate oxidative-stress related disorders. As for plants in general, the antioxidant potential of sage and thyme species have mostly been assessed for phenolic-enriched extracts [1,65,171]. These compounds can combat oxidative stress conditions and thus possess others health beneficial activities (e.g. anti-inflammatory) protecting against related diseases [1,47,158,159]. In these context, in recent years there has been an effort to gather scientific data to corroborate the traditional usage of these plants and its application in food industry, namely in the development of natural additives and safe food products [1,2,14,65]. Important literature gathered on this topic is resumed in Table 5.

The most common methods to screen the antioxidant properties of plants are based on the (i) scavenging of stable free radicals such as DPPH$^{\bullet}$ (2.2-diphenyl-1-picrylhydrazyl), ABTS$^{\bullet}$ (2.2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), $^{\bullet}OH$, $O_2^{\bullet -}$ and NO$^{\bullet}$; ii) the reduction of metal ions from the higher to the lower oxidation state by reducing power method (RP) and ferric reducing antioxidant power assay (FRAP); iii) the oxygen radical absorbance capacity (ORAC) that measures the radical chain breaking ability of antioxidants by the inhibition of peroxyl radical ($RO_2^{\cdot}$)–induced oxidation and trolox equivalence antioxidant capacity (TEAC); iv) the inhibition of lipid peroxidation by ß-carotene bleaching test; and v) the inhibition of lipid peroxidation by decreasing the thiobarbituric acid reactive substances (TBARS). The extensive use of such assays is mainly attributed to their simplicity and quickness of execution [172,173]. As shown in Table 5, several *Salvia* and *Thymus* species have been screened for their antioxidant capacities through chemical methods. Most studies present the antioxidant activity expressed as EC_{50} values (half maximal effective concentration), which is equivalent to sample concentration that provides 50% of antioxidant activity. Note that, in some cases, the antioxidant activity is also displayed as Trolox Equivalents (TE) per dry weight (DW).

- **Antioxidant activity of *Salvia* plants**

Among sage plants, *S. officinalis* is the most widespread worldwide and also the most studied regarding antioxidant capacity [174]. Due to its high antioxidant capacity, *S.*

officinalis phenolic extracts are also frequently used as a reference to assess antioxidant properties of other less investigated plants [66,175]. DPPH EC_{50} values of distinct extracts from this species were established in the range of 1.98 and 10.1 µg/mL [66,176,177], being close to that of the flavonol quercetin (2.79 µg/mL) [178]. The antioxidant activity of *S. officinalis* phenolic-rich extracts has also been extensively assessed by the $ABTS^{\bullet}$ scavenging assay (EC_{50}= 4.79-50.8 µg/mL) [176,178], through evaluation of the ability to scavenge superoxide (EC_{50}= 5.3-10.1 µg/mL) [177] and hydroxyl radicals (EC_{50}= 1.36 µg/mL) [176], and also by the ORAC assay (0.4-1.8 mmol TE/100 mL) [179]. Moreover, metanolic extracts and decoctions of *S. officinalis* were shown to exhibit very low EC_{50} values, as estimated by ß-carotene bleaching (EC_{50}=6.6 and 50.9 µg/mL, respectively) and TBARS assays (EC_{50}= 2.1 and 10.4 µg/mL, respectively) [180].

Overall, recent data concerning phenolic-rich extracts of other *Salvia* species also highlight their promising potential, among which *S. nemorosa* and *S. sclarea* represent the most studied species [10,73,74,175,181]. In this context, Kostic et al [74], reported good antioxidant ability of ethanolic extracts of *S. sclarea* by $DPPH^{\bullet}$ (EC_{50}=27.0 µg/mL) and inhibition of ß-carotene (EC_{50}= 19.1 µg/mL) methods, the latter being promising than that of rosmarinic acid (EC_{50}= 32.6 µg/mL). In addition, methanolic extracts of *S. nemorosa* and *S. sclarea* collected in Iran, showed $DPPH^{\bullet}$ EC_{50} values in the range of 82 to 191 µg/mL [10,73]. In addition, Jeshvaghani et al. [175] exhibited the potency of these two extracts, presenting EC_{50} values about 1.4–2.2 lower than BHT (3.5-di-tert-butyl-4-hydroxytoluene), used as standard reference solution. These two species exhibited antioxidant potential based in other chemical *in vitro* tests, namely ORAC and ABTS assays [181].

Several other polar extracts from different populations of *Salvia* plants possess antioxidant potential. In this regard, methanolic extracts of *Salvia* from Tunisia origin (*S. aegyptiaca, S. argentea* and *S. verbenaca*), were shown to scavenge $DPPH^{\bullet}$ (EC_{50}=21-77 µg/mL) and $ABTS^{\bullet}$ (141-318 µM TE/mg DW), as well as to reduce the ferric form (81-205 mM Fe(II)/mg DW) [66]. Other several *Salvia* plants have shown to be good antiradicalar sources. *E.g.* DPPH EC_{50} values of methanolic: chloroform (1:1) extracts of 14 *Salvia* plants from South Africa, which in general contained rosmarinic acid as major phenolic constituent, were shown to vary between 1.6 µg/mL (*Salvia schlechteri*) and 74.2 µg/mL (*Salvia garipensis*) [38]. In the same line, polar extracts of *S. amplexicaulis* [106] and *S. ringens* [114], both rich in kaempferol glycosides, were suggested as good antioxidant agents, as estimated by $DPPH^{\bullet}$, $ABTS^{\bullet}$ and FRAP methods. In addition, phenolic-rich extracts from different populations of *S. fruticosa* [108] and aqueous and

methanolic extracts of *S. cadmica* from Turkey [71], were demonstrated to scavenge DPPH• and ABTS• and reduce the ferric ion as well (Table 5).

In good agreement with these results, other *Salvia* polar extracts, such as *S. amplexicaulis* [106], *S. halophila* [76], *Salvia virgata*, *Salvia persica*, *Salvia cereal* and *S. reuterana* [175], showed inhibition of the β-carotene bleaching capacity, in some cases equals those of the standard compounds. Similary, the potential of *Salvia* species to inhibit lipid peroxidation by TBARS was reported for *S. halophila*, with inhibition percentage equal to reference commercial compound BHT [76].

Table 5 – Antioxidant properties of phenolic-enriched extracts of distinct *Salvia* species

Salvia species	Origin	Solvent extraction (major components)	Results of screen assay	Ref
S. aegyptiaca	Tunisia	MeOH (RA, caffA deriv, Api-7-Glc, Lut-7-Glc, Nar, DT)	**DPPH** (EC_{50}, µg/mL): 21.1-22.6 / **FRAP** (mM Fe(II)/mg): 149-164 / **ABTS** (µM TE/mg dry plant): 312-318, collection site	[66]
S. amplexicaulis	Lithuania	EtOH, H_2O, CO_2 (ND)	**ORAC** (µM TE/g DW): 4735 (EtOH), 676 (H_2O), 1914 (CO_2) / **ABTS** (µM TE/g DW): 1177 (EtOH), 79.5 (H_2O)	[181]
S. amplexicaulis	Macedonia	DCM, EA (Hyper, Coumarin) H_2O, EtOH, MeOH (RA, Lut- 5-O-Glc, Kam-3-O-acetil Glc-7-O-Rhm)	**DPPH** (EC_{50}, µg/mL): 27.8 (EtOH), 15.8 (H_2O), 15.1 (MeOH), 196.5 EA, 593.2 (DCM), 5.1 (AA) / **ABTS** (mg AAE/g): 2.8 (EtOH), 2.8 (H_2O), 2.4 (MeOH), 0.7 EA, 0.4 (DCM), 2.8 (BHT) / **FRAP** (µmol Fe(II)/g)= 979 (EtOH), 1372 (H_2O), 1178 (MeOH), 112 EA, 117 (DCM), 445 (BHT) / **β-carot bleach** (%): 40.4 (EtOH), 41.8 (H_2O), 13.3 (MeOH), 58 (BHT)	[106]
S. argentea	Tunisia	MeOH (RA, caffA deriv, Api-7-Glc, Lut-7-Glc, Nar, DT)	**DPPH** (EC_{50}, µg/mL)= 33.9-77.1 / **FRAP** (mM Fe(II)/mg)= 81.6-105 / **ABTS** (µM TE/mg)= 141-173, collection site	[66]
S. bicolor	Egypt	MeOH (PrcA, CouA, GA, Lut-7-O-Glc)	**DPPH** (EC_{50}, µg/mL) = 321 (MeOH), 250 (GA)	[128]
S. cadmica	Turkey	EA, MeOH, H_2O (RA, caffA, Lut, Api)	**DPPH** (µmol TE/g dry plant)= 5.9 (EA), 54.7 (MeOH), 40.5 (H_2O) / **CUPRAC** (µmol TE/g dry plant)= 20.9 (EA), 50.9 (MeOH), 54.5 (H_2O) / **ABTS** (µmol TE/g dry plant)= 7.1 (EA), 84.9 (MeOH), 102.2 (H_2O)/ **FRAP** (µmol TE/g dry plant)= 13.5 (EA), 80.0 (MeOH), 98.0 (H_2O)	[71]
S. fruticosa	Greece: (populations 1 and 2)	MeOH (RA, ChlgA, caffA)	**FRAP** (µM Trolox/g DW): 31.83-202.93; 135.81-326.22 / **ABTS** (µM Trolox/g DW): 60.94-242.3; 199.64-312.49 / **DPPH** (µM Trolox/g DW): 182.99-192.62; 185.30-192.19, populations 1 and 2, respetively	[108]
S. halophila	Turkey	Hexane (ND), EA (RA, CaffA, Lut), MeOH (RA, GA, Lut-Glc), MeOH 50% (CaffA, *o*-coumaric, *p*-OH-BA, Lut-Glc), H_2O (RA)	**DPPH** (EC_{50}, mg/mL)= 0.4 (EA), 0.6 (50% MeOH), 1.0 (H_2O, MeOH) / **FRAP** (AAE mmol/g extract)= 1.0 (EA, 50% MeOH), < 1.0 (H_2O, MeOH, Hexane), 0.025 (GA) / **β-carot bleach** (AA%)= 80 (Hexan), 65 (EA), < 40 (MeOH, 50% MeOH, H_2O), 90 (BHT) / **Iron (II) Thiocyanate** (%)= 91 (hexan), 89 (EA), 70 (50% MeOH), 68 (H_2O), 18 (MeOH), 90 (BHT), at 1% conc / **TBARS** (%)= 94 (hexan), 91 (H_2O), 82 (50% MeOH), 80 (EA), 52 (MeOH), 90 (BHT), at 1% conc.	[76]

S. miltiorrhiza	Commercial	H_2O (ND), 95% EtOH (ND)	**DPPH** (EC_{50}, µg/mL): 202 (H_2O), 105 (EtOH)	[171]
S. miltiorrhiza	China	H_2O (Danshensu, SA A, SA B)	**DPPH** (EC_{50}, µg/mL)= 131/ **ORAC** (µmol/g) = 757	[183]
S. nemorosa	Lithuania	EtOH, H_2O, CO_2 (ND)	**ORAC** (µM TE/g DW): 2392 (EtOH), 718 (H_2O), 1193 (CO_2) / **ABTS** (µM TE/g DW): 1005 (EtOH), 150 (H_2O)	[181]
S. nemorosa	Iran	80% MeOH (ND)	**DPPH** (EC_{50}, µg/mL)= 138.4 (80% MeOH), 1.79 (quercetin)	[10]
S. nemorosa	Iran	MeOH (RA, caffA, Que)	**DPPH** (EC_{50}, µg/mL)= 82 (MeOH), 64 (BHT)	[73]
S. nemorosa	Iran	MeOH (ND)	**DPPH** (EC_{50} µg/ml): 473 (MeOH), 211 (BHT) / **β-carot bleach** (%): 20 (MeOH), < 20% (BHT), at 500 µg/ml	[175]
S. officinalis	Iran	MeOH (ND)	**DPPH** (EC_{50} µg/ml): 233 (MeOH), 211 (BHT) / **β-carot bleach** (%): 79 (MeOH), < 20% (BHT), at 500 µg/ml	[175]
S. officinalis	Algerian	MeOH-H_2O (RA, Flav)	**DPPH** (EC_{50} µg/ml): 1.98 / **ABTS** (EC_{50} µg/ml): 4.79 / **OH scav** (IC_{50} µg/ml): 1.36	[176]
S. officinalis	Portugal	DE, EA, *n*-but, H_2O (ND)	**DPPH** (EC_{50} µg/ml): 3.9 (EA), 4.1 (*n*-but), 2.8 (H_2O) / **O_2- scav** (EC_{50} µg/ml): 5.5 (DE), 5.3 (EA), 8.9 (*n*-but) 10.1 (H_2O)	[177]
S. officinalis	Spain	H_2O inf, H_2O dec, 80% MeOH (RA, Lut-O-Glc, Lut-O-Glr)	**DPPH** (EC_{50} µg/ml): 96 (H_2O inf), 75.5 (H_2O dec), 33 (80% MeOH) / RP (IC_{50} µg/ml): 84 (H_2O inf), 66.5 (H_2O dec), 25 (80% MeOH) / **β-carot bleach** (EC_{50} µg/ml): 139 (H_2O inf), 50.9 (H_2O dec), 6.6 (80% MeOH) / **TBARS** (EC_{50} µg/ml): 18.0 (H_2O inf), 10.4 (H_2O dec), 2.1 (80% MeOH)	[180]
S. officinalis	Lithuania	CO_2, EtOH, H_2O	**ORAC** (µM TE/g DW): 2535 (EtOH), 1143 (H_2O), 6015 (CO_2) / **ABTS** (µM TE/g DW): 1080 (EtOH), 225 (H_2O)	[181]
S. officinalis	Commercial	H_2O, MeOH (RA, SA K isomer, Lut-Hex, Api-Glr)	**DPPH** (EC_{50}, µg/ml)= 69 (MeOH), 2.79 (Que) / **ABTS** (EC_{50} µg/ml): 19.9 (MeOH), 50.8 (H_2O), 1.17 (Que)	[178]
S. officinalis	Commercial	H_2O (RA and deriv)	ORAC (mmol TE/100mL): 0.4 to 1.8	[179]
S. officinalis	Tunisia	MeOH (RA, caffA deriv, Api-7-Glc, Lut-7-Glc, Nar, DT)	**DPPH** (EC_{50}, µg/mL): 3.4-10.1 / **FRAP** (mM Fe/mg): 179-197/ **ABTS** (µM TE/mg): 645-766, collection site	[66]

S. palaestina	Jordan	*n*-but (8"-O-(methyldihydrocafeoyl) Rsm-methyl-3-O-methyl-Rsm)	**DPPH** (EC_{50}, µg/mL): 3.01 (salpalaestinin); 1.0 (methyl-3-O-methylRsm), 1.1 (BHA)	[150]
S. ringens	Macedonia	EtOH (Kam-3-O-Glu-7-O-Rhm, rutin); MeOH (RA, Kam-3-O-Glu-7-O-Rhm, rutin); DCM (Hyperoside); EA (Lut, Kam-3-O-Glu-7-O-Rhm)	**DPPH** (EC_{50}, µg/mL): 17.3 (EtOH), 20.3 (MeOH), 266.2 (DCM), 22.2 (EA), 5.1 (AA) / **ABTS** (mg AAE/g): 2.44 (EtOH), 1.19 (MeOH), 0.58 (DCM), 2.36 (EA), 2.8 (BHT) / **FRAP** (µmol Fe(II)/g): 1088.3 (EtOH), 274.8 (MeOH), 191.1 (DCM), 969.8 (EA), 180 (AA)	[114]
S. sclarea	Lithuania	EtOH, H_2O, CO_2 (ND)	**ORAC** (µM TE/g DW): 3820 (EtOH), 867 (H_2O), 570 (CO_2) / **ABTS** (µM TE/g DW): 1634 (EtOH), 153 (H_2O)	[181]
S. sclarea	Iran	80% MeOH (ND)	**DPPH** (EC_{50}, µg/mL)= 190.7 (80% MeOH), 1.79 (quercetin)	[10]
S. sclarea	Iran	MeOH (ND)	**DPPH** (EC_{50} µg/ml): 290 (MeOH), 211 (BHT) / **β-carot bleach** (%): 35 (MeOH), < 20% (BHT), at 500 µg/ml	[175]
S. sclarea	Serbia	EtOH (RA, Lut-7-glc, Api-7-Glc)	**DPPH** (EC_{50} µg/ml): 27.0 (EtOH), 6.1 (RA), 2.4 (BHA) / **β-carot bleach** (EC_{50} µg/ml): 19.1 (EtOH), 32.6 (RA), 0.03 (BHA)	[74]
S. verbenaca	Tunisia	MeOH (RA, caffA deriv, Lut, Nar, DT)	**DPPH** (EC_{50} µg/mL): 23-36.3 / **FRAP** (mM Fe(II)/mg): 106-159 / **ABTS** (µM TE/mg): 165-205, collection site	[66]
S. virgata	Iran	MeOH (ND)	**DPPH** (EC_{50} µg/ml): 198 (MeOH), 211 (BHT) / ↓**β-carot bleach** (%): 95 (MeOH), < 20% (BHT), at 500 µg/ml	[175]
Other *Salvia* species				
S. cereal S. reuterana, S. persica	Iran	MeOH (ND)	**DPPH** (EC_{50} µg/ml): 598 (*S. reuterana*), 883 (*S. cereal*), 1810 (*S. persica*), 211 (BHT) / **β-carot bleach** (%): 30 (*S. reuterana*), 55 (*S. cereal*), 95 (*S. persica*), < 20% (BHT), at 500 µg/ml	[175]

S. aethiopis, S. atropatana, S. eremophila, S. hypoleuca, S. limbata, S. santolinifolia, S. syriaca, S. xanthocheila	Iran	80% MeOH (ND)	**DPPH** (EC_{50}, µg/mL)= 89.5 (*S. atropatana*) - 557 (*S. limbata*), 1.79 (quercetin)	[10]
S. africana-caerulea, S. africana-lutea, S. albicaulis, S. aurita, S. chamelaeagnea, S. disermas,S. dolomítica, S. garipensis, S. lanceolata, S. muirii, S. namaensis, S. radula, S. repens, S. runcinata, S. schlechteri, S. stenophylla	South Africa	MeOH:$CHCl_3$ (RA)	**DPPH** (EC_{50}, µg/mL): 1.61 (*S. schlechteri*), 74.2 (*S. garipensis*), >100 (*S. dolomitica, S. radula*), 11.1-68.1 (Others) / **ABTS** (EC_{50}, µg/mL)= 11.9 (*S. muirii*), 69.3 (*S. radula*), 14.6-50 (Others)	[38]

AA– ascorbic acid; AAE - ascorbic acid equivalent; ABTS– 2.2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay; Api – apigenin; BA – benzoic acid; BHA - 2(3)-t-butyl-4-hydroxyanisole; BHT - 3.5-di-*tert*-butyl-4 hydroxytoluene; CaffA/CaffA deriv– caffeic acid/caffeic acid derivative; ChlgA - chlorogenic acid¸β-carot bleach– β-carotene/linoleic acid bleaching inhibition assay; CouA - Coumaric acid; CUPRAC– cupric ion reducing antioxidant capacity; Deriv– derivatives; DCM-dichloromethane; DE - diethyl ether; dec – decoction; DPPH– 2.2-diphenyl-1-picrylhydrazyl radical scavenging assay; DT – diterpenes; DW– dry weight; EA- ethyl acetate; EtOH- ethanol; Hex – hexoside; FA - ferulic acid; Flav – flavonoids; FRAP– ferric reducing antioxidant power; GA – gallic acid; GAE– gallic acid equivalents; Glc–glucoside; Glr - glucuronide; Hyper – Hyperoside; inf – infusion; Kam – kaempferol; LA perox– linoleic acid peroxidation; Lut – luteolin; Lut – luteolin; MeOH- methanol; Nar – naringin; *n*-but - *n*-butanol; ND– not determined; OH scav– hydroxyl radical scavenging assay; O_2- scav– O_2- radical scavenging assay; ORAC– oxygen radical absorbance capacity; p-OH-BA – p-hydroxybenzoic acid; PrcA - Protocatchuic acid; QA - quinic acid; Que – quercetin; RA– rosmarinic acid; Rhm - rhamnoside; RP – reducing power; Rsm - rosmarinat; SA - salvianolic acid; TBARS– thiobarbituric acid reactive substances assay; TE– trolox equivalent.

- **Antioxidant activity of *Thymus* plants**

The antioxidant activity of *Thymus* species has also been extensively assessed and its phenolic content and/or composition have closely associated to their antioxidant properties [81], as well as to their health benefits [1]. In more detail, water, methanolic and hydroalcoholic extracts of *T. vulgaris*, i.e., the most investigated *Thymus* species, DPPH• radical EC_{50} values have been reported in the range of 1.8 and 44.7 µg/mL [45,176,178]. Moreover, phenolic-rich extracts of *T. vulgaris* were shown to be good ABTS• (EC_{50}= 0.7-13.8 µg/mL) [176,178] and OH• (EC_{50}= 0.24 µg/mL) [176] scavenging agents.

In the last years, the antioxidant activity of distinct thyme extracts has been investigated with promising results. In particular, a methanolic extract of *T. nummularius*, also called *Thymus pseudopulegioides*, was claimed to have higher DPPH• scavenging ability (EC_{50}= 5.7 µg/mL) than BHT (EC_{50}= 47.1 µg/mL), and 1.5 times better than in both ABTS and ß-carotene bleaching inhibition methods [83]. Besides that, high radical scavenging ability was demonstrated for water extracts of three *Thymus* species (*Thymus carnosus, Thymus camphoratus* and *T. mastichina*) originated from Portugal, presenting low EC_{50} values for DPPH• (EC_{50}= 3.2-3.9 µg/mL) and for superoxide radical (EC_{50}= 9.5-13.6 µg/mL) [177]. Moreover, hydromethanolic extracts of *T. capitatus* [84] and *T. mastichina* [81], which presented rosmarinic acid as major phenolic constituent, were shown to have high antioxidant potential based in DPPH (18- 149 mg TE/g DW) and FRAP assays (30–154 mg TE/g DW). In the same line, aqueous extracts of *Thymus atlanticus, Thymus satureioides* and *T. zygis*, were also evaluated for the DPPH• scavenging ability and for the capacity in reducing iron, whose results were equal or better than the reference commercial compound [54,184]. In the same line, Kindl et al [45] reported the antioxidant potencial of ethanolic extracts of six *Thymus* species from Croatia, namely *Thymus longicaulis, T. praecox subsp. polytrichus, T. pulegioides, T. serpyllum* subsp. *serpyllum, Thymus striatus* and *T. vulgaris,* which were shown to possess low EC_{50} values (µg/mL) for DPPH (3.01-6.01), RP (11.4-15.1) and TBARS methods (34.3 -80.0).

Commonly, the antiradicalar/antioxidant activity of *Salvia* and *Thymus* extracts has been positively correlated to their phenolic content and to the presence of specific phenolic compounds [66,81,106,108], which frequently appear as major components of its polar extracts (e.g. rosmarinic acid, caffeic acid, apigenin7-*O*-glucoside, luteolin-7-*O*-glucoside) [66,71,74,76,81,83,84,106,108,180,184]. Amongst them, rosmarinic acid is frequently the principal phenolic acid in the said extracts and it is believed to play a central role in their antioxidant abilities [66,74,81,83,106,108]. In fact, this caffeic acid

dimer was shown to be sixtheen folder better than α-tocopherol [83], twice as high than trolox [45], and 0.4 times higher than the BHA (2(3)-t-butyl-4-hydroxyanisole) [74], in DPPH radical-scavenging assay. Rosmarinic acid has also higher reducing power capacity (EC_{50}= 2.7 μg/mL) than trolox (EC_{50}= 6.6 μg/mL) [45] and higher $ABTS^{\bullet}$ scavenging ability than BHT [83]. Besides that, this caffeic acid is also known to prevent lipid peroxidation, as reported by Ertas et al. [83], whose EC_{50} value was 1.2-fold higher than BHT in the ß-carotene bleaching inhibition assay.

Moreover rosmarinic acid, some of its derivatives together with other non-flavonoids (e.g. danshensu) and flavonoid compounds (e.g. luteolin, apigenin, quercetin and their glycosides) might also positively contribute to the antioxidant capacity of these plant extracts (Table 6) [1,2,65,174]. In fact, salvianolic acid B, salvianolic acid A and litospermic acid, were demonstrated to be the main phenolic compounds contributing to the high antioxidant capacity of a traditional Chinese medicine formula enriched in danshen, as assessed by the DPPH method (EC_{50} values of 8.80, 12.1 and 15.6 μM respectively) [183]. In addition to caffeic and/or rosmarinic acid derivatives, flavonoids (in particular luteolin, apigenin and its derivatives) might also contribute to the antioxidant capacity of *Salvia* and *Thymus* species (Table 6), since they are good antiradicalar/antioxidants as evaluated in chemical and cellular models. Namely, luteolin and apigenin (50 μg/mL) were demonstrated to drastically decrease the levels of intracellular ROS under oxidative-stress conditions on a hepatic cellular model [185]. Moreover, luteolin, was shown to exhibit high antioxidant activity, being 2.3 and 1.5 folder better than trolox in DPPH assay-scavenging and in RP method, respectively [45]. Furthermore, it was demonstrated to effectively prevent lipid peroxidation events, exhibiting an EC_{50} value of 2.03 μg/ml in the TBARS assay, which is ten-times lower than that of rosmarinic acid [45].

Table 6 – Antioxidant properties of phenolic-enriched extracts of distinct *Thymus* species

***Thymus* plants**	**Origin**	**Solvent extraction (Major components)**	**Results of screen assay**	**Ref**
T. atlanticus	Morocco	H_2O (RA, caffA, Que)	**DPPH** (EC_{50}, µg/ml)= 120 (H_2O), 510 (trolox) / **FRAP** (mmol trolox/g extract)= 40.0 (H_2O), 44.3 (trolox)	[184]
T.camphoratus	Portugal	DE, EA, *n*-but, H_2O (ND)	**DPPH** (EC_{50} µg/ml): 3.1 (DE), 2.7 (EA), 6.4 (*n*-but), 3.2 (H_2O)/ **O_2- scav** (EC_{50} µg/ml): 7.8 (DE), 11.0 (*n*-but), 9.5 (H_2O)	[177]
T. capitatus	Greece	70% MeOH (RA, FA, Nar, Lut); H_2O (RA, Caff, Epi, Epig)	**DPPH** (mg TE/g DW): 56.2 / **ABTS** (mg TE/g DW): 75.2 / **FRAP** (mg TE/g DW): 76.1	[84]
T. carnosus	Portugal	DE, EA, *n*-but, H_2O (ND)	**DPPH** (EC_{50} µg/ml): 4.0 (DE), 3.0 (EA), 5.2 (*n*-but), 3.6 (H_2O) / **O_2- scav** (EC_{50} µg/ml): 12.3 (DE), 8.9 (EA), 8.9 (*n*-but), 13.6 (H_2O)	[177]
T. longicaulis	Croatia	EtOH (ND)	**DPPH** (EC_{50} µg/ml): 3.01 (EtOH), 0.66 (RA),0.73 (Lut), 1.67 (Trolox) / **RP** (EC_{50} µg/ml): 11.8 (EtOH), 2.67 (RA), 4.51 (Lut), 6.64 (Trolox) / **TBARS** (EC_{50} µg/ml): 34.3 (EtOH), 21.1 (RA), 2.03 (Lut)	[45]
T. longicaulis	Italy	EtOH-H_2O (RA, SA K, Lut-O-hex, Quer-O-Hex)	**DPPH** (EC_{50}, µg/mL): 9.5 (H_2O-MeOH), 5.1 (trolox) / **ABTS** (EC_{50}, µg/mL): 9.5 (H_2O-MeOH), 5.1 (trolox) / **RP** (µM TE/g extract): 475 (H_2O-MeOH) / **ORAC** (µM TE/g extract): 776.5 (H_2O-MeOH)	[186]
T. mastichina	Portugal	DE, EA, *n*-but, H_2O (ND)	**DPPH** (EC_{50} µg/ml): 2.7 (DE), 3.7 (EA), 4.0 (*n*-but), 3.9 (H_2O) / **O_2- scav** (EC_{50} µg/ml): 10.0 (DE), 4.9 (EA), 6.9 (*n*-but), 12.2 (H_2O)	[177]
T. mastichina	Portugal	MeOH, H_2O (ND)	**DPPH** (EC_{50} mg/ml): 0.69 (MeOH), 2.57 (H_2O), 0.04 (trolox) / **RP** (EC_{50} mg/ml): 0.23 (MeOH), 0.7 (H_2O), 0.03 (trolox) / **β-carot bleach** (EC_{50} mg/ml): 0.9 (MeOH), 0.003 (trolox) / **TBARS** (EC_{50} mg/ml): 0.43 (MeOH), 0.004 (trolox)	[44]
T. mastichina	Spain	50% MeOH (RA, CaffA, Lut, Lut-Glc)	**DPPH** (mg TE/g DW): 18-149 / **FRAP** (mg TE/g dw): 30 – 154, different populations	[81]
T. nummularius	Turkey	MeOH (QA, RA, Lut, Kam)	**DPPH** (EC_{50} µg/ml): 5.73 (MeOH), 1.21 (RA), 47.1 (BHT), 19.6 (α-Toc) / **ABTS** (EC_{50} µg/ml): 7.1 (MeOH), 1.7 (RA), 10.9 (BHT) / **β-carot bleach** (EC_{50} µg/ml): 6.54 (MeOH), 12.1 (RA), 9.95 (BHT)	[83]

T. pulegioides	Portugal	MeOH (ND)	**DPPH** (EC_{50} µg/ml): 680 / **RP** (EC_{50} µg/ml): 490 / **β-carot bleach** (EC_{50} µg/ml): 30 / **TBARS** (EC_{50} µg/ml): 220	[43]
T. pulegioides	Croatia	EtOH (ND)	**DPPH** (EC_{50} µg/ml): 4.18 (EtOH), 0.66 (RA), 0.73 (Lut), 1.67 (Trolox) / **RP** (EC_{50} µg/ml): 11.4 (EtOH), 2.67 (RA), 4.51 (Lut), 6.64 (Trolox) / **TBARS** (EC_{50} µg/ml): 34.8 (EtOH), 21.1 (RA), 2.03 (Lut)	[45]
T. satureioides	Morocco	H_2O (RA, caffA, Quer)	**DPPH** (EC_{50}, µg/ml)= 440 (H_2O), 510 (trolox) / **FRAP** (mmol trolox/g extract)= 40.1 (H_2O), 44.3 (trolox)	[184]
T. vulgaris	Croatia	EtOH (ND)	**DPPH** (EC_{50} µg/ml): 5.6 (EtOH), 0.66 (RA),0.73 (Lut), 1.67 (Trolox) / **RP** (EC_{50} µg/ml): 14.1 (EtOH), 2.67 (RA), 4.51 (Lut), 6.64 (Trolox) / **TBARS** (EC_{50} µg/ml): 69.6 (EtOH), 21.1 (RA), 2.03 (Lut)	[45]
T. vulgaris	Algerian	MeOH-H_2O (RA, Flav)	**DPPH** (EC_{50} µg/ml): 1.78 / **ABTS** (EC_{50} µg/ml): 0.69 / **OH scav** (EC_{50} µg/ml): 0.24	[176]
T. vulgaris	Commercial	H_2O, MeOH (RA, SA K isomer, Lut-Hex, Eri-Glc)	**DPPH** (EC_{50}, µg/ml): 44.7 (MeOH), 2.79 (Que) / **ABTS** (EC_{50} µg/ml): 13.8 (MeOH), 49.6 (H_2O), 1.17 (Que)	[178]
T. zygis	Morocco	H_2O (RA, caffA, Quer)	**DPPH** (EC_{50}, µg/ml): 440 (H_2O), 510 (trolox) / **FRAP** (mmol trolox/g extract): 65.0 (H_2O), 44.3 (trolox)	[184]
Other *Thymus* species				
T. praecox subsp. polytrichus, T. serpyllum subsp. serpyllum, T. striatus	Croatia	EtOH (ND)	**DPPH** (EC_{50} µg/ml): 3.4 (*T. praecox*), 4.06 (*T. striatus),* 6.01 (*T. serpyllum*) 0.73 (Lut), 1.67 (Trolox) / **RP** (EC_{50} µg/ml): 15.1 (*T. praecox*), 14.7 (*T. striatus),* 14.5 (*T. serpyllum*), 2.67 (RA), 4.51 (Lut), 6.64 (Trolox) / **TBARS** (EC_{50} µg/ml): 78.7 (*T. praecox*), 63.0 (*T. striatus),* 80.0 (*T. serpyllum*), 21.1 (RA), 2.03 (Lut)	[45]

AA– ascorbic acid; AAE - ascorbic acid equivalent; ABTS– 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay; Api – apigenin; BHA - 2(3)-t-butyl-4-hydroxyanisole; BHT - 3.5-di-tert-butyl-4 hydroxytoluene; CaffA/CaffA deriv– caffeic acid/caffeic acid derivative; β-carot bleach– β-carotene/linoleic acid bleaching inhibition assay; Deriv– derivatives; DCM-dichloromethane; DE - diethyl ether; dec – decoction; DPPH– 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay; DW– dry weight; EA- ethyl acetate; Epi - epicatechin; Epig - epigallocatechine; Eri – eriodictyol; EtOH- ethanol; Hex – hexoside; FA - ferulic acid; Flav – flavonoids; FRAP– ferric reducing antioxidant power; Glc–glucoside; Glr - glucuronide; Kam – kaempferol; Lut – luteolin; MeOH- methanol; Nar – naringin; *n*-but - *n*-butanol; ND– not determined; OH scav– hydroxyl radical scavenging assay; O_2- scav– O_2- radical scavenging assay; ORAC– oxygen radical absorbance capacity; Que – quercetin; RA– rosmarinic acid; Rhm - rhamnoside; RP – reducing power; SA - salvianolic acid; TBARS– thiobarbituric acid reactive substances assay; TE– trolox equivalente.

1.3.2. Anti-inflammatory activity

Inflammation is the first biological response of the immune system against tissue injury or infection, that involves both localized and systemic effects. Tissue damage induces the first mechanism of immune recognition (innate immunity), which involves physical, chemical, and biological barriers, specialized cells and soluble molecules, such as plasma enzyme mediators by the kinin, clotting and fibrinolytic systems [187,188]. This unspecific response allows the recognition of pathogens via a series of conserved microbial motifs, known as pathogen associated molecular patterns (PAMPs), through pathogen-recognition receptors (PRRs) present in immune cells. Toll-like receptors (TLRs) are a class of important PRRs, that could recognize PAMPs such as bacterial endotoxin LPS (lipopolysaccharide), being the TLR4 one of the well-known. When activated, PRRs triggers a series of signaling cascades that culminate in the activation of nuclear transcription factors such as nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1). Such factors regulate the expression of a series of target genes involved in inflammation, namely chemokines and pro-inflammatory cytokines. The latter include interleukins (e.g. IL-1β, IL-6, IL-8, IL-12), interferon (INF-γ) and tumor necrosis factor (TNF-α), that in turn stimulate and amplify the inflammation, inducing many of the localized and systemic changes peculiarly of inflammatory response [161,187,189]. The activation of adherent molecules by chemokines and complement split products is also triggered, enabling leukocytes migration into tissue spaces and the release of inflammatory mediators, which will culminate in changes on the vasculature of the damaged tissue, vasodilatation, vascular permeability and release of soluble mediators and extravasation of fluids. This process results in cellular influx and migration of antigen presenting cells from the periphery to the lymphoid organs activating the adaptive immunity development [161,187,189]. In turn, the breakdown of phospholipids in the membrane of immune cells are degraded into lyso-platelet activation factor to yield platelet activation factor (PAF) and arachidonic acid, whose metabolism generate prostaglandins and tromboxanes by the cyclooxygenases pathway and leukotrienes by lipoxygenase pathway, stimulated by the enzymes cyclooxygenases (constitutive COX-1 and inducible COX-2) and by 5-lipoxygenase (5-LOX), respectively [188,190,191].

Oxidative stress is also an important factor in the triggering and progression of inflammation, occuring overproduction of ROS and RNS, in which the enzyme NO synthase (iNOS) plays an important role, contributing to tissue damage [161,187]. Overall, these oxidative agents result from activation of phagocytic cells, whilst nonphagocytic cells can also produce reactive species in response to proinflammatory cytokines. Moreover, recent findings have shown that the costimulation of TLR produces oxidative stress with unbalance of proinflammatory cytokine production [161].

Despite the protective role of inflammation, when acute inflammation is not resolved and lasts longer or gets deregulated, a chronic inflammation emerges, which is characterized by progressive degeneration of the tissue and activation of the adaptive immune system [187,188]. In attempting to repair the damaged tissue, T cells produce cytokines such as INF-γ and TNF-α, thus activating specifically macrophages, developing in chronic inflammation. This condition has a detrimental effect in several diseases including atherosclerosis, rheumatoid arthritis, infection, asthma, obesity, diabetes, neurodegenerative diseases, cancer and inflammatory bowel disease [192,193]. Thus, the proinflammatory stimulus (cytokines or microbial antigens) bind to the cell receptors triggering the NF-kB or mitogen-activated protein kinase (MAPK) signaling cascades, culminating in transcription of several proinflammatory genes encoding biochemical mediators, such as cytokines and enzymes [187,188]. Several molecules involved in these cascades events are the target of anti-inflammatory and immunomodulatory drugs.

Drug-based therapies employing corticosteroids and a variety of nonsteroidal anti-inflammatory drugs (with greater specificity for the COX-2 enzyme or inhibition of 5-LOX) also have side effects [188,191,194]. In addition, since inflammation can be counteracted by anti-inflammatory cytokines (IL-4, IL-10, IL-13), transforming growth factor (TGF)-β and specific receptors for IL-1 and TNF-α [187], several drugs have been developed to inhibit cytokine activity (e.g. inhibitors of TNF-α and IL-1) commoly used to control chronic inflammatory diseases [195]. Thus, it seems to be important to replace the use of immunomodulators with natural compounds that mimic their effects, focusing on the development of alternative and innovative therapeutics for the treatment of these diseases.

A growing body of experimental evidence suggests that plant extracts or their bioactive compounds (e.g. phenolic compounds) have potencial therapeutic application in the treatment of several inflammatory diseases [1,47]. *In vitro* and *in vivo* studies suggest that the anti-inflammatory activity of plants might occur through distinct mechanisms, including the inhibition of pro-inflammatory cytokines, intercellular adhesion molecules and enzymes (e.g. iNOS, COX-2, 5-LOX), as well as, transcription factors, such as NF-κB [19,187,192,195–197]. Therefore, biological activity of some enzymes or other inflammatory products has been accessed *in chemico* (e. g. inhibition of LOX-5 and COX activity) [38,177,198]. In addition, inflammatory molecules could be also measured by enzyme-linked immunosorbent assay (ELISA), flow cytometry and/or Western Blot methods [199–202], and its gene expression could be performed by analysis by real-time polymerase chain reaction (RT-PCR) [186,201]. Cell cultures methods were also used for studying the potential of natural products to counteract distinct mechanisms of inflammation (cytokines, enzymes, ROS or NO levels), after LPS and/or IFN-γ stimulation of human monocytic leukemia cell line (THP-1 cells) or, more

frequently, on monocyte/macrophage cell line RAW 264.7, simulating the acute inflammation process. Griess reaction is vastly used for measuring the nitrite accumulation in the culture supernatant on monocyte/macrophage cell line RAW 264.7 [171,196,203].

- **Anti-inflammatory activity of *Salvia* and *Thymus* plants – *in vitro* methods**

The anti-inflammatory potential of sage and thyme plants has been extensively assessed (Table 7). In particular, the capacity to inhibit enzymes involved in inflammation was evaluated for a water extract of *S. officinalis* and a methanolic extract of *Salvia radula*, which were demonstrated to be potent inhibitors of 5-LOX (EC_{50} values of 27.6 and 78.8 µg/mL, respectively) [38,177]. In addition, ethanolic and methanolic extracts of different *Salvia* species presented inhibitory capacity of LOX, with the highest values registered for *Salvia sericeo-tomentosa* var. *hatayica* (74%), followed by *Salvia freyniana* (66%) [77]. Notably, the activity of 5-LOX was also retarded by *Thymus* polar extracts (Table 7), namely from *T. camphoratus, T. carnosus* and *T. mastichina* (EC_{50}= 27-53 µg/mL) [177]. Moreover, inhibition of COX-1 was reported for an aqueous extract of *Salvia sclareoides* [204] and for a hydroalcoholic extract of *S. officinalis* [198], in a concentration dependent manner (EC_{50} values of 271 and 19 µg/ml, respectively).

On other hand, the anti-inflammatory potential of sage and thyme genera, was measured *in chemico* by the ability of scavenging $NO^{\bullet}$. In particular, ethanolic extracts of six *Thymus* species showed EC_{50} NO• scavenging results 1-3 times higher than the tested standard compound (Trolox), whith the best results being obtained for the *T. pulegioides* and *T. longicaulis* species (EC_{50} of 69.8 and 71.6 µg/mL, respectively) [45]. Moreover, ethanolic extracts of *S. plebeia* (rich in caffeic acid, luteolin-*O*-7-glucoside and hispidulin) were shown to exhibit higher $NO^{\bullet}$ scavenging than quercetin (60% and 80% of inhibition, respectively, at 200 µg/mL) [205].

Studies on cellular models also support the hypothesis that *Salvia* and *Thymus* phenolic-enriched extracts possess anti-inflammatory activity. In this sense, ethanolic and methanolic extracts of *S. plebeia*, were shown to suppress iNOS and prostaglandin E_2 (PGE_2) expression, ROS and NO levels in LPS-activated RAW264.7 macrophage cells in a concentration-dependent manner [196,206]. Likewise, aqueous extracts of *S. miltiorrhiza* were demonstrated to effectively decrease the levels of NO and iNOS expression on LPS-activated mouse macrophages [171,207]. In particular, *S. miltiorrhiza*, downregulates NO and TNF-α production (EC_{50}= 200 and 900 µg/mL, respectively) without affecting the cell viability of LPS-stimulated macrophages [171]. Moreover, an ethanolic extract of *S. plebeia* (100 µg/ml) was able to suppress NO level (about 30% of inhibition) in LPS-activated RAW264.7 macrophage cells [196]. Besides, an hydroalcoholic extract of *T. longicaulis*, with high amounts of rosmarinic

acid, was shown to inhibit COX-2 gene expression in THP-1 macrophages [186], and the aquoues extracts of *S. officinalis* and *T. vulgaris* were able to decrease the release of the cytokine IL-8 for both H_2O_2 or TNFα-stimulated peripheral blood lymphocytes (PBL) [200]. Likewise, *T. vulgaris* was shown to inhibit the production of cytokines IL-1β and TNF-α in LPS-activated RAW 264.7 macrophages after exposure to different concentrations of propylene glycol extracts [208].

Notably, some authors emphasized a straight correlation between the high levels of phenolics and/or flavonoids in the extract with its antioxidant/anti-inflammatory activities [171,200]. Among non-flavonoids, rosmarinic acid (the major phenolic in *Salvia* and *Thymus* plants in general) and its derivatives are probably positive contributors to anti-inflammatory activities, being able of counteracting proinflammatory cytokines and the enzymes COX-2 and iNOS in a dose-dependent manner in LPS-induced inflammatory cells, as well as, by downregulating expression of NF-κB promoter-related genes [202,209,210]. Moreover, ethyl rosmarinate, a structurally modified rosmarinic acid, was claimed to be a potent inhibitor against lung inflammation, based on NO and PGE_2 inhibition and also by decreasing of mRNA and protein expression of iNOS and COX-2 in LPS-induced alveolar macrophages [209]. In addition, the anti-inflammatory potential of lithospermic acid extracted from root of *S. miltiorrhiza* was demonstrated by inhibition of the production and mRNA expression of cytokines TNF-α, IL-1β, and IL-6, and by downregulation of proteins involved in the signaling NF-kB-pathway, in stimulated THP-1 macrophages [211].

Similarly, flavonoids have also been shown to inhibit the expression of various enzyme systems, inflammatory mediators (e.g. molecules adhesion, cytokines and chemokines) and molecules involved in signal transduction pathways implicated in the inflammatory response [47,212]. In particular, luteolin (a flavone present in *Salvia* and *Thymus* plants in general), was demonstrated to inhibit the expression of COX2 and TNF-α secretion in IFN-γ-stimulated RAW264.7 macrophage cells, and to inhibit the phosphorylation of signal transducer and activator of transcription (STAT)-3, a crucial transcription factor of proliferation and activation of T cells [199].

- **Anti-inflammatory activity of *Salvia* and *Thymus* plants – *in vivo* methods**

The anti-inflammatory effect of sage and thyme species were observed *in vivo* models as well. Experimental animal models of acute inflammation comprise the paw edema induced by carrageenan, histamine, dextran and/or formalin; ear edema induced by toxics as croton oil, arachidonic-acid, oxazolone, phorbol myristate acetate and xylene; measurement of myeloperoxidase in leucocytes, total numbers of polymorphonuclear leukocytes (LPM); vascular permeability induced by acetic acid in tissues from animal models [196,201,207,213].

Other *in vivo* studies include chronic inflammation models, namely cotton pellet-induced granuloma and glass rod granuloma [190,213].

Among the reported bibliography of *Salvia* plants, significant inhibition of paw edema (21-28%) was reported after treatment with extracts obtained from *S. virgata* and *S. halophila* (at 100 mg/kg) or *Salvia splendens* (at 1000 mg/kg) [115,214], which were rich in RA. Similar inhibition of paw edema (35%) was observed after treatment with a methanolic extract obtained from *S. bicolor* (at 50 mg/kg), which was particularly enriched in the flavones luteolin-7-*O*-glucoside and apigenin [128]. Moreover, oral administration of an ethanolic extract from *S. sclarea* in a rat model of periodontal disease, was able to decrease cytokines production IL-6, IL-1β and TNF-α, possibly due to its high content of rosmarinic acid (165.3 μg/mg of extract) [74]. In addition, *Salvia przewalskii* extract, rich in rosmarinic acid and salvianolic acid B, improved urinary protein excretion in a rat model of immune complex glomerulonephritis, increasing total serum protein and serum albumin values [215]. These findings showed that *S. przewalskii* extract treatment could maintain renal function effectively, displaying its anti-inflammatory potential, since inflammatory response is the characteristic feature of glomerular disease in immune glomerular injury [215].

As for *Thymus* plants, Khouya et al [184] found that aqueous extracts of *T. atlanticus, T. satureioides and T.zygis* could suppress croton oil-induced mice ear edema (at 900 μg/ear) and *T. atlanticus,* and *T. zygis* also inhibited carrageenan-induced rat paw edema (at 50 mg/kg), with similar potency to that of the non-steroidal anti-inflammatory drug indomethacin. These authors showed that a water extract of *T. zygis* could suppress croton oil-induced mice ear edema in 70% (at 900 μg/ear) and inhibit carrageenan-induced rat paw edema in 3.7% (at 50 mg/kg), comparing to indomethacin [54,184].

It is possible that major phenolic constituents of sage and thyme extracts might contribute to their anti-inflammatory effects since either rosmarinic acid, caffeic acid, luteolin-7-*O*-glucoside and apigenin were previously reported as to effectively counteract inflammatory events *in vivo* models [201,202,216,217]. In particular, rosmarinic acid suppressed LPS-induced proinflammatory cytokine [201,202] and inhibited the expression of COX-2 and iNOS protein in mice disease -inflammatory models [202,218]. The overall data highlighs the anti-inflammatory potential of *Salvia* and *Thymus* polar extracts throught distinct mechanisms, also emphasizing their potential to be used in the development of alternative drugs for the treatment of a variety of diseases settled on oxidative and/or inflammatory events, including infectious diseases, diabetes, cancers, ageing and others.

Table 7 – Anti-inflammatory properties of phenolic-enriched extracts of distinct *Salvia* and *Thymus* species

Plant Species	**Origin**	**Solvent extraction (Major**	**Screen Assay**	**Effect**	**Ref**
Salvia species					
S. bicolor	Egypt	MeOH (Lut-7-O-Glr, Api)	Carrageenan-induced paw edema	At 50 mg/kg after 2 h: ↓18.1%, after 3h: ↓29.4%, after 4 h: ↓35.4%	[128]
S. fruticosa	Lebanon	MeOH (Lut, Rut, GA)	Carrageenan-induced paw edema	At 200 mg/kg after 4 h: ↓50% (aerial parts), 44% (roots)	[219]
S. halophila	Turkey	MeOH (RA, caffA)	Carrageenan-induced paw edema	At 100 mg/kg after 4.5 h: ↓21%, after 6 h: 20%	[214]
S. miltiorrhizae	Commer cial	H_2O (ND)	LPS- or IFN- γ-activated RAW 264.7 macrophages / Inhibition of NO and TNF-α production	NO (EC_{50}, mg/mL) = 0.2 TNF-α (EC_{50}, mg/mL)= 0.9	[171]
S. officinalis	Commer cial	H_2O (ND)	IL-8 release of PBLs prior to stimulation by TNFα and H_2O_2	↓IL-8 release in 25 and 35% upon stimulation of TNF-α and H_2O_2, respectively	[200]
S. officinalis	Brazil	EtOH-H_2O (ND)	Croton Oil-induced ear edema MPO activity	At 5 mg/ear: ↓ear edema by 34.1% At 5mg/ear: ↓MPO activity by 46%	[220]
S. officinalis	Portugal	DE, EA, *n*-but, H_2O (ND)	5-LOX activity	5-LOX (EC_{50} µg/mL): 29.6 (DE), 41.0 (EA), 39.3 (*n*-but), 27.6 (H_2O)	[177]
S. plebeia	Korea	EtOH (ND)	Acetic acid-induced vascular permeability Carrageenan-induced inflammation in the air pouch Total numbers of LPM in the air pouches NO production in LPS-induced RAW264.7 macrophages	At 50, 100 and 200 mg/kg: ↓ vascular permeability of 3.5%, 22.9% and 66.4%, respectively At 0.1, 0.3 and 1.0 mg/pouch: ↓ exudate volume of 21.9%, 27.1% and 42.7%, respectively (in comparison to dexamethasone (0.01 mg/pouch: ↓ exudate 60%) At 0.1, 0.3 and 1.0 mg/pouch: ↓ LPM 4.9%, 14.6% and 32.7%, respectively At 10, 50 and 100 µg/ml: ↓ NO of 50, 25 and 15 µM, respectively	[196]
S. plebeia	China	EtOH (Pld C, Plf)	NO production in LPS-induced RAW264.7 macrophages	EC_{50} (µM)= 105 and 127	[203]
S. radula	South Africa	MeOH (RA)	5-LOX inhibithy	EC_{50} (µg/mL)= 78.8	[38]

S. sclarea L.	Serbia	EtOH (RA, Lut-7-*O*-Glc, Api-7-*O*-Glc)	Cyokines measurement in rats' gingival tissues	↓ of TNF-α, IL-1β, and IL-6	[74]
S. splendens	Egypt	80% MeOH (RA, caffA deriv, flav glyc)	Carrageenan-induced paw edema	At 1000 mg/kg after 2 h: ↓28% (potency 0.55 in comparison to indomethacin)	[115]
S. sclareoides	Portugal	H_2O (RA, Lut-di-Glr, Lut-acetyl-Glc)	COX-1 inhibithy	EC_{50} (µg/mL)= 271.0	[204]
S. virgata	Turkey	MeOH (RA, *o*-CouA)	Carrageenan-induced paw edema	At 100 mg/kg after 4,5 h: ↓28%, after 6 h: ↓24%	[214]
Thymus species					
T. atlanticus	Morocco	H_2O (RA, caffA, Que)	Croton oil-induced mice ear edema Carrageenan-induced rat paw edema	At 900 µg/ear after 8 h: ↓84.6% (in comparison to indomethacin) At 50 mg/kg after 5 h: ↓9.5% (in comparison to indomethacin)	[184]
T. camphoratus (*T.cm*), *T. carnosus* (*T.cr*)	Portugal	DE, EA, *n*-but (ND)	5-LOX inhibithy	5-LOX (EC_{50} µg/ml): 29.9 (DE), 27.4 (EA), 28.0 (*n*-but), *T.cm*; 23.5 (DE), 29.6 (EA), 18.3 (*n*-but), *T.cr.*	[177]
T. longicaulis	Italy	EtOH-H_2O (RA, SA K, Lut-O-Hex, Quer-O-Hex)	COX-2 gene expression on THP-1 cells	At 50 µg/ml: ↓42% (extract collected in October)	[186]
T. mastichina	Portugal	DE, EA, *n*-but (ND)	5-LOX inhibithy	5-LOX (EC_{50} µg/ml): 62.5 (DE), 53.1 (EA), 30.5 (*n*-but)	[177]
T. satureioides	Morocco	H_2O (RA, Lut-7-Glc, hesp)	Croton oil-induced mice ear edema	At 900 µg/ear after 8 h: ↓29.7% (in comparison to indomethacin)	[184]
T. vulgaris	Commer cial	H_2O (ND)	IL-8 release of PBLs prior to stimulation by TNFα and H_2O_2	↓IL-8 release in 35 and 37% upon stimulation of TNF-α and H_2O_2, respectively	[200]
T. vulgaris	Commer cial	PG (ND)	Cytokines production (pg/mL) by in LPS-induced RAW264.7 macrophages	IL-1β (pg/ml) = 28, 2, 2 at 25, 50 and 100 mg/mL extract, respectively TNF-α (pg/ml)= 4466, 824, 12 at 25, 50 and 100 mg/mL extract, respectively	[208]
T. zygis	Morocco	H_2O (RA, caffA, Lut-7-O-Glc)	Croton oil-induced mice ear edema Carrageenan-induced rat paw edema	At 900 µg/ear after 8 h: ↓70% (in comparison to indomethacin) At 50 mg/kg after 5 h: ↓3.7% (in comparison to indomethacin 10 mg/kg)	[54,184]

Api– apigenin; CaffA/CaffA deriv– caffeic acid/caffeic acid derivatives; CouA – coumaric acid; COX-2– cyclooxygenase; DE - diethyl ether; EA - ethyl acetate; EO– essential oil; EtOH– ethanol; Flav/flav glyc– flavones/flavone glycosides; GA – gallic acid; Glc–glucoside; Glr – glucuronide; Hesp – hesperetin; Hex – hexoside; IFN-γ– interferon-γ; IL–interleucin; 5-LOX– 5-lipoxygenase; LPM - polymorphonuclear leukocytes; LPS– lipopolysaccharide; Lut – luteolin; MeOH– methanol; MPO – myeloperoxidase; n-but - n-butanol; ND – not determined; NF-κB – nuclear factor-kappa B; NO– nitric oxide; PBL - peripheral blood lymphocytes; PG - Propylene glycol; PGE2 - prostaglandin E2; Pld C - plebeiolide C; Plf – plebeiafuran; Que – quercetin; RA– rosmarinic acid; Rut – rutin; SA - salvianolic acid; THP-1 - human monocytic leukemia cell line; TNF-α– tumor necrosis factor-α.

1.3.3. Anticancer activity

Cancer is a major health problem worldwide being the second leading cause of death globally and is estimated to account for 9.6 million death in 2018 [221]. The genesis of tumor can be diverse, namely, chronic inflammation, some infectious diseases (e.g. virus infection), radiation, environmental and chemical agents and genetic predisposition, has long been recognized as a risk factor for the development of tumors in many different tissues [222–224].

The cancerous state develops when the normal tissue homeostasis is broken, resulting in an imbalance between cellular proliferation and cell death. A number of oncoproteins and tumor suppressors involved in regulation of these processes are often aberrant in cancer, thereby promoting cell proliferation and inhibition of programmed cell death (apoptosis). Indeed, cellular oncogenes products arise from malignantly transformed cells and can involve mutations, DNA amplifications or translocations, overall increasing or decreasing expression of gene products, involved in cellular proliferation or cell death [222,225–227].

Apoptosis can be induced by activation of the caspase cascade through death receptors pathway or the mitochondrial pathway, although others several signaling pathways are critically involved in the regulation of programmed cell death [225,226]. The Bcl-2 family of proteins have important roles in governing mitochondrial death signaling, whose members have either proapoptotic (Bax, Bad) or antiapoptotic (Bcl-2, Bcl-XL) [228]. Thus, the induction of programed cell death in cancer cells is one of the strategies used in the development of anti-cancer drugs [229–231]. Recent reports showed that a new class of small regulatory RNA molecules, termed microRNAs (miRNAs), can modulate these major proliferation pathways through interacting with critical cell cycle regulators, which have been proposed as novel oncogenes or tumor suppressors [225,226,232,233]. In addition, is it presently accepted that chronic inflammation promotes oxidative events that contribute to magliant transformation of cells associated with tumor cell proliferation and tumor progression, as well as, angiogenesis and tissue remodeling, induction of neoplastic transformation and metastasis [163,167]. In these context, a notably body of experimental evidence suggests that pharmacologic attenuation of inflammatory processes, can restore the cancer cells to an apoptosis-permissive and growth-inhibitory state [163,168].

Considering the high antioxidant, inflammatory and/or anticancer properties of diverse plants, recent researches have demonstrated that secondary metabolites can be effective regulators of the cell cycle of tumor cells at an early stage by targeting specific cell signaling molecules, leading to apoptosis or cellular senescence and/or to act at later stages of tumorigenesis by inhibition of angiogenesis or prevention of tumor invasion and metastasis [227,232,234–236]. In this context, plants used in traditional forms have been the focus of attention as plant-based

foods, whose regular consumption correlated with a lower incidence of cancer, because are rich in phenolic acids and flavonoids with promising chemopreventive and chemotherapeutic potentials for cancer prevention and/or treatment [227,232–236]. In many cases, the effects of natural compounds are due to the inhibition of the activity of different transcriptional factors, including the key factors NF-kB and AP-1, involved in the expression of several inflammation mediators [227,232,235,236].

- **Cell cytotoxicity assays**

Salvia and *Thymus* plants have been widely studied as potential sources of chemotherapeutic agents, particularly *in vitro* cellular models. As for drugs in general, the majority of these studies have used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as a first approach method to estimate cytotoxicity/growth-inhibitory activity of extracts in cancer cell lines (Table 8). Note that MTT is a colorimetric method that measures the activity of mitochondrial enzymes that reduce MTT to purple formazan, whose conversion is often used as a estimative of viable cells [178,229]. Similar principle methods are commonly used, including those based on the reduction of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) [186,237] and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulphophenyl)-2H-tetrazolium inner salt) [238,239] which result in the formation of the colored formazan; or sulforhodamine B (SRB) assay that is based on the ability of SRB to bind to protein components of cells, which have been fixed to tissue-culture plates by trichloroacetic acid [240]. Most studies present the antioxidant activity expressed as IC_{50} values (half inhibition concentration), which is equivalent to sample concentration that inhibited 50% of the cell grow.

In general, the anticancer activity of *Salvia* and *Thymus* extracts have been mostly accessed for *S. officinalis, S. miltiorrhiza* and *T. vulgaris* and mainly based on cytotoxic assays (Table 8). Using MTT method, Brahmi et al. [176] reported that hydroalchoolic extracts of *S. officinalis* were able to inhibit the viability of neuroblastoma and hepatoblastoma HepG2 cells lines, showing dose dependent cytotoxic effects after 48 h exposure. Using the same assay, crude methanolic extract of *S. officinalis* showed cytotoxic effect against non-Hodgkin's B-cell lymphoma (Raji), human leukemic monocyte lymphoma (U937), human acute myelocytic leukemia (KG-1A), human breast carcinoma (MCF-7), human prostate cancer (PC3), and mouse fibrosarcoma (WEHI-164) cell lines in a dose-dependent manner, as reflected by the comparatively low IC_{50} values (range between 142 and 179 µg/ml) to that obtained in Human Umbilical Vein Endothelial Cells (HUVEC, IC_{50} values exceeding 600 µg/mL) [241]. In addition, the cytotoxicity effect of Danshen alcohol, extracted from *S. miltiorrhiza*, was proved on human oral squamous carcinoma (OSCC) cell lines HSC-3 and OC-2 (IC_{50} values of 27 and 31 µg/ml,

respectively) [230]. In the same way, methanol extracts of *S. miltiorrhiza* roots, showed reduced cells survival in a dose-dependent manner on human lung cancer Glc-82, A549 and H460 cells, with respectively IC_{50} values of 21, 61 and 75 µg/mL [231].

Extracts of other *Salvia* species have been shown to display cytotoxic capacity on several tumoral cell lines, including the methanolic extracts of *Salvia eremophila* (IC_{50} of 12, 16 and 48 µg/ml on human promyelocytic leukemia HL60, human erythromyeloblastoid leukemia K562 and breast cancer MCF-7 cells, respectively) [10]. Moreover, in this study, the authors also described that both methanol and dichloromethane (DCM) extracts of *S. aegyptiaca, Salvia aethiopis, Salvia hypoleuca*, *Salvia limbata* and *Salvia santolinifolia* were active against the tested cell lines, suggesting that compounds with different polarities must be involved in their cytotoxic activity [10]. In addition, Alimpic et al. [106,114] reported IC_{50} values in the range 9.8 to 164 µg/mL for aqueous and ethanolic extracts of *S. amplexicaulis* and *S. ringens* (both rich in kaempferol glycosides), towards colon carcinoma HCT-116 cell line.

Based on MTS assay, Jiang et al. [238] reported IC_{50} values of 84.0 and 75.8 µg/ml ethanolic extracts from leaves and roots of *S. officinalis,* respectively, towards HepG2 cells. Both extracts disclosed less anti-proliferation ability in normal human liver cells (WRL-68), with higher IC_{50} values (>100 µg/ml), which further proved the anticancer property of leaves and roots extracts of *S. officinalis* and the safety to normal human liver cells (WRL-68) [238]. Cytotoxicity effect by the same method was also demonstrated by methanolic extracts of *S. chloroleuca*, together with *n*-hexan and DCM fractions, that showed a viability decrement of human breast cancer cell line MCF-7, in a concentration-dependent manner (IC_{50} of 60, 28 and 25 µg/mL, respectively) [239].

As regard to the anticancer effects of *Thymus* plants, despite most of previous works have been focus on *T. vulgaris,* some other species have also been suggested as good antiproliferative agents (Table 8). E.g. methanolic extracts of *T. schimperi*, rich in the flavone luteolin and derivatives, required doses of 50–100 µg/mL to inhibit human gastric adenocarcinoma AGS cells growth by 50% (IC_{50}= 88 µg/mL) [147]. In addition, Galasso et al. [186] showed that *T. longicaulis* extracts, containing high amounts of quercetin-*O*-hexoside, luteolin-*O*-exoside and salvianolic acid K, caused a decrease of cell viability on breast cancer MDA-MB-23, colon carcinoma HCT-116 and lung fibroblast MRC-5 cells, equal to or higher than those observed for vinblastine, a known anti-cancer agent [186]. Besides, Esmaeili-Mahani et al. [229] reported that polar extracts of *Thymus caramanicus* (at 150-200 µg/mL), could reduce the viability of human breast adenocarcinoma MCF-7 for about 60% and interestingly, its concomitant treatment (40 and 80 µg/mL extract) with the vincristine effectively potentiated the effect of the anticancer drug.

Moreover, based on MTT methods, commercial aqueous extracts of *T. vulgaris* and *T. serpyllum* were shown cytotoxic effect towards adriamycin-resistant human breast cancer cell line (MCF-7/Adr), with IC_{50} values of 407 and 399 µg/mL, respectively [242]. Amongst representative compounds of the extratcts (caffeic acid, rosmarinic acid, lithospermic acid, luteolin-7-*O*-glucuronide, luteolin-7-*O*-rutinoside and eriodictiol-7-*O*-rutinoside), rosmarinic acid and lithospermic acid were the most active. Notably, plant extracts (including *T. vulgaris* and *T. serpyllum*) proved to be more toxic against the cancer resistant cells than the analysed phenolics (that exhibited greater toxicity against the non-resistant cells), suggesting the existence of synergistic or additive effects among the extracts components.

Finally, components of *S. miltiorrhiza*, including danshensu and salvianolic acids, were previously demonstrated to be exhert anticancer properties, both *in vitro* and *in vivo* models [243,244]. Similarly, tanshinones (tanshinone IIA, cryptotanshinone and tanshinone I), identified as major components of the methanol extract of the dried root of *S. miltiorrhiza* Bunge, is known to be one of the contributors to the anticancer properties of this plant [231].

- **Tumor-cell membrane integrity and DNA-protective evaluation**

Cell membrane integrity was generally used to determine the level of metabolically active cells, such as the activity of lactate dehydrogenase (LDH) and cell ATP quantity, the latter based on the fact that ATP is present in all metabolically active cells and can be a marker for cell viability and proliferation [238]. The anticancer properties of ethanolic extracts from leaves and roots of *S. officinalis* and ethanolic extracts from roots of *S. miltiorrhiza* was confirmed by LDH release and cellular ATP decrease when HepG2 cells were treated with different concentrations of extract, which resulted in a significant dose-dependent reduction in cellular proliferation related with untreated control cells [238]. In another study, *S. miltiorrhiza* polysaccharides (50 µg/mL) induced LDH leakage on LoVo colorectal carcinoma cell line, being more effective than 5-fluoro-2,4(1H,3H)-pyrimidinedione (100 µg/mL), a typical antitumor drug [234]. These results indicated that these sage extracts had significant cytotoxic effect on these cancers cells lines, and the inhibitory effect might have originated from inducing cell membrane damage.

DNA repair ability is also important, as DNA damage can lead to carcinogenesis if replication proceeds without proper repair. The DNA-protective effects of plant extracts against DNA strand breaks induced by oxidant agents, hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), has been performed by the comet assay, based in single cell gel electrophoresis [178,245]. In fact, exposure for 24 h of H_2O_2-induced human colon carcinoma Caco-2 and human cervical cancer HeLa cells to aqueous extracts of *S. officinalis* and *S. fruticosa* resulted in a protective effect on DNA damage [245]. This effect was also observed

for the two major phenolic constituents of the extracts (rosmarinic acid and luteolin-7-*O*-glucoside) thereby highlighting their intervention in this mechanism. The same authors also reported promising results in assays of DNA repair, as evaluated in oxidative-induced Caco-2 cells. In this experimental conditions, the independent treatment with both extracts (50 µg/mL for 24h), as well as with luteolin-7-*O*-glucoside (20 µM), were able to increase the rate of rejoining of DNA strand breaks [245]. Notably, Kosics et al. [178] reported that the protective effects of *S. officinalis* and *T. vulgaris* (rich in rosmarinic acid) against DNA damage induced by strong oxidants (H_2O_2 and DMNQ), achieved the highest DNA-protective capacity at concentrations of 2 and 0.5 mg/ml, respectively [178].

- **Apoptosis, cell cycle and angiogenesis evaluation**

Besides the decrement of cell viability of cancer cells, important effects of *Salvia* and *Thymus* extracts in key mechanisms of tumor formation/progression (e.g. apoptosis and angiogenesis) have also been attested. The apoptosis of proliferative cells after treatment with plant extracts has been screened *in vivo* and *in vitro* models, being the flow cytometry method extensively used for this purpose [231,234,238]. The most potent extracts/drugs treatment result in an increased number of tumoral cells undergoing apoptosis due to their accumulation in the early phases of the cell cycle, taking into account the sequential occurrence of sub-G1, G1, S, G2, culminating in the final mitosis (phase M) [169]. In particular, 43% of HCA-7 cells arrested in the G1 phase after treatment with ethanolic extracts of *S. officinalis* (16 µg/GAE/mL), while 45% of cells arrested in these cell cycle phase after treatment with celocoxib (50 µM) [169]. Consistent with these results, after treatment with *S. miltiorrhiza* polysaccharides (at 100 and 400 µg/mL), the percentage of human colon cancer LoVo cells in S phase were significantly increased (50% and 44%, respectively) in comparison with 32% of the untreated cells, probably by blockade of the the tumor cell transition from S to G2/M phase [234]. In addition, hepatocellular carcinoma HepG2 cells treated with ethanolic and acetone extracts of *S. miltiorrhiza* and *S. officinalis*, induced significantly cellular apoptosis at early and/or late stage/necrotic [238].

As a result of cell cycle arrest, DNA replication and protein synthesis in tumor cells could not be complete, leading to the disturbed cell cycle progression that causes the death of the tumor cells. Detection of molecules involved in cell proliferation or in apoptosis, kinases involved in several signaling pathways, oncogenes and tumor suppress activity can be evaluated by flow cytometry and western blot analysis [229–231,238]. Gene expression analysis of these biological components can also be performed by molecular methods such as RT-PCR [186,201].

Several studies have investigated the apoptosis induction of cancer cells through activation of the caspase cascade or by disruption in the balance between pro- and antiapoptotic Bcl-2 family members, after exposure to sage and thyme extracts. Thus, the potential mediators of plant extracts induced cell damage can be analyzed by caspase cascade activation (e.g cleaved caspases) and by the expression of Bcl-2 family members, as cell apoptosis markers [169,229]. In particular, treatment of human lung cancer cells Glc-82 with methanolic extract of *S. miltiorrhiza* (20 and 40 μg/ml) showed a significant increase in the levels of cleaved caspase-9 and caspase-3, as well as, up-regulation the expression levels of pro-apoptotic Bax and down-regulation the expression of the Bcl-2 and Bcl-xl compared to control cells [231]. Moreover, treatment of human oral squamous carcinoma (OSCC) cell lines HSC-3 for 48 h with Danshen alcohol extract gradually increased the caspase-3 apoptotic pathway [230]. Apoptotic capacity was also registered for aqueous extracts of *T. vulgaris*, which were shown to increase the activity of caspase3/7 on human colon carcinoma cells HCT116 [246]. In addition, Esmaeili-Mahani et al. [229] highlited the anticancer activity of *T. caramanicus* hydroalcoohlic extracts, based on their ability to induce apoptosis in human breast cancer cells MCF-7 through the increment of caspase-3 and Bax and decreasing of Bcl-2 expression. These *T. caramanicus* extracts (200 - 250 μg/ml) also potentiated the cytotoxicity of vincristine, which was accompanied by an increase in cleaved caspase-3 [229].

Another characteristic event of cell apoptosis is the fragmentation of genomic DNA into multiples units producing a typical characteristic ladder on agarose gel electrophoresis. This event was observed in the human breast carcinoma MCF-7 cells, within 48 h after treatment with DCM solvent fraction of *S. chloroleuca* at 12.5, 50, 100 and 200 μg/mL [239].

The effects of *Salvia* and *Thymus* compounds on the activity of different transcriptional factors, associated to the expression of tumor suppressors have also been investigated. In this context, methanolic extracts of *S. miltiorrhiza* were shown to downregulate the PI3K/Akt signaling pathways of human lung cancer cells Glc-82 [231]. These events resulted in an increased expression levels of the tumor suppressors p53 and p21 (4.2 and 3.4 times higher than those of untreated control cells, respectively) [231]. In addition, *S. officinalis* and *S. fruticosa* aqueous extracts (particularly enriched in rosmarinic acid and luteolin derivatives) were demonstrated to induce apoptotic cell death in rat insulinoma m5F and/or colon adenocarcinoma HCT15 and colon carcinoma CO115 cell lines. This effect was concentration-dependent for the two latter cell lines [247,248] and it was observed for rosmarinic acid (at 100 μM dose) as well. Authors correlated, at least in part, the activity of sage extracts/rosmarinic acid to the inhibition of MAPK/ERK pathway.

The effects reported above were also investigated for individual compounds present in *Salvia* and *Thymus*, highlighting their relevance in the anticancer properties of these plants.

Rosmarinic acid has been implicated in inhibition of apoptotic factors (e.g. Bax and caspases 3/ 9) [217], TLRs (e.g. TLR4) and transcriptional factors such as NF-κB, which regulate a number of target genes involved in cell proliferation, survival and angiogenesis in inflammatory diseases [201,202,217,218]. In turn, flavonoids have been claimed as cancer protective agents, e.g. quercetin was shown to produce cell cycle arrest in proliferating lymphoid cells and growth-inhibitory effects on several malignant tumor cell lines [47].

Since inhibition of angiogenesis has been recognized to be advantageous for prevention of inflammation and neoplastic growth, invading distant tissues forming metastasis [227,249], *Salvia* and *Thymus* species have been investigated with respect to their antiangiogenic activity. In this context, non-toxic concentrations of ethanolic extracts of *S. officinalis* were shown to effectively inhibit proliferation and cell migration (two important mechanisms for new blood vessels formation) of human umbilical vein endothelial cells (HUVECs) [250]. As *S. officinalis* is enriched in rosmarinic acid, it is probable that this phenolic compound can play a role in that protective mechanism. In fact, Huang and colleagues [251] have stressed the ability of this caffeic acid dimer in inhibiting several important steps of angiogenesis including proliferation, migration, adhesion and tube formation of HUVEC.

In vivo studies have also reported the tumor growth inhibition and antiangiogenic capacity of extracts from sage and thyme origin. In particular, ethanolic extracts of *S. officinalis* (at 100–300 μg/mL) were shown to inhibit the capillary tube formation in rat aorta models [250]. In addition, the chick chorioallantoic membrane (CAM) angiogenesis model was used for assessing an inhibitory activity of the ethanol extract of *S. plebeia* on vascular development [196]. Moreover, *S. miltiorrhiza* methanolic extracts (10, 20, 40 mg/kg) were demonstrated to reduce tumor volume and weight of tumor xenografts in nude mice when compared to normal saline control, with growth inhibition rates of 36.7, 46.9 and 60.8, respectively [231]. In that study, TUNEL assay which enable to observe DNA fragmentation resultant from apoptosis, showed an increment of apoptotic cells after treatment with *S. miltiorrhiza* extracts [231]. Likewise, Wang et al. [230] demonstrated the reduction of tumor growth in mice treated with Danshen alcohol extract compared to the control group.

- **Molecules involved in inflammation**

A growing body of evidence suggests that the antitumoral activity of plant natural products are related to their anti-inflammatory and antioxidant activities, which in turn are claimed to be associated with their phenolic composition [227,235,236]. In this sense, the activity of enzymes involved in inflammation, such as COX-2 and its product PGE-2 (known as carcinogenesis promoters), those involved in enhancing detoxification, like as GPX, SOD or catalase [232,235], and cytokines or others immunological mediators, can also be used for evaluation

of the antitumoral activity of *Salvia* and *Thymus* extracts [169,178,186]. In more detail, an extract obtained from commercially-available *S. officinalis* was shown to increase TNF-α secretion by macrophages [247]. Note that this is an important substance present in tumor microenvironments to counteract tumor cell survival and metastasis, as they act as pro-inflammatory mediators, thus allowing macrophages to destroy cancer cells. On other hand, Moon et al. [252] found that rosmarinic acid could increase TNF-α-induced apoptosis in human leukemia U937 cells. Furthermore, ethanolic extract of *S. officinalis* decreased COX-2 expression (40% at 40 μg GAE/mL) and consequently released PGE-2, and/or inhibited the growth and viability of colorectal cancer HCA-7 cell line, as well [169]. Likewise, hydroalcoholic extracts of *T. longicaulis*, particularly rich in rosmarinic acid and methylapigenin, exhibited a strong anti-inflammatory and anticancer effectiveness through inhibition of COX-2 gene expression on human leukemia cell line THP-1 macrophages [186]. These results suggest that there downregulation of COX-2 expression/activity and growth inhibition of tumor cells are closely associated [169,186], determining the relationships of anti-inflammatory, antioxidant and cytotoxic potential.

Table 8 – Anticancer properties of phenolic-enriched extracts of distinct *Salvia* and *Thymus* species

Plant Species	Origin	Solvent extraction (Major components)	Screen Assay	Effect	Ref
Salvia species					
S. amplexicaulis	Macedonia	H_2O, EtOH, MeOH (RA, Lut-5-O-Glc, Kam-3-O-(6´´-O-acetil-Glu)-7-O-Rhm)	Cytotoxic effect (MTT) on HCT-116 cells	MTT (IC_{50}, µg/mL)= 164 (EtOH), 114 (H_2O), 0.02 (5-Fl), for 24h/ 582 (EtOH), 161 (H_2O), 0.8 (5-Fl), for 72h	[106]
S. chloroleuca	Iran	MeOH, DCM, H_2O, *n*-hex (ND)	Cytotoxic effect (MTS), apoptosis on MCF-7 cells	MTS (IC_{50}, µg/mL)= 60 (MeOH), 25 (DCM), >250 (H_2O), 28 (*n*-hex), for 48h / apoptosis (G1-peak): ↑ (DCM) after 12, 25 and 50 µg/mL extract for 48h, apoptosis (DNA fragmentation): ↑ (DCM) after 12, 50, 100 and 200 µg/mL extract for 48h	[239]
S. eremophila	Iran	DCM, MeOH, 80% MeOH (ND)	Cytotoxic effect (MTT) on HL60, K562, MCF-7 cells	MTT (IC_{50}, µg/mL)= HL60: 11 (DCM), 12 (MeOH), 25 (80% MeOH), 0.8 (Cis); K562: 16 (DCM), 16 (MeOH), 43 (80% MeOH), 2.1 (Cis) / MCF-7 Cells: 46 (DCM), 48 (MeOH), 75 (80% MeOH), 6.9 (Cis)	[10]
S. miltiorrhiza	Commercial	EtOH, EtOH-H_2O (ND)	Cytotoxic effect (MTT) on OSCC (HSC-3 and OC-2 cells)/ markers of apoptosis (flow cytometry and western blot) / *In vivo* antitumor growth	MTT (IC_{50}, µg/mL)= HSC-3: 40 (EtOH), 47 (EtOH-H_2O) after 24h; HSC-3: 27 (EtOH); OC-2: 31 (EtOH) after 48h / Flow cytometry= HSC-3: ↑ caspase 3, at 10, 25, or 50 μg/mL, after 48h; change in mitochondrial membrane potential ($\Delta\psi$) / western blot: unchange of bcl-2, Bcl-xl, Bax and Bad / *in vivo*: ↓ mice tumor volumes= 1056 mm^2 and 552 mm^2, at 50 mg/kg and 100 mg/kg extract, respectively	[230]
S. miltiorrhiza	China	MeOH (Tsh IIA, crytsh, Tsh I)	Cytotoxic effect (MTT) on lung cancer Glc-82 cells/ Clonogenic survival in Glc-82 cells/ cell cycle and apoptosis (flow cytometry and western blot) / *In vivo* antitumor growth (TUNEL assay)	MTT (IC_{50}, µg/mL) = Glc-82: 21; A549: 61; H460: 75, after 24h / Clonogenic survival (% cell colonies rate): 65, 45, 30 at 10, 20, and 40 μg/mL of extract, respectively; Flow cytometry = Glc-82 cells´s cell cycle: ↓ G_0/G_1 phase, G_2/M arrest, at 40 µg/ml extract, after 24h; apoptotic cells (%): 6, 10 and 31 at 10, 20 and 40 μg/mL of extract, respectively; Western blot = Glc-82 cells: ↑ caspase-9, ↑ caspase-3 at 20 and 40 μg/mL of extract; ↑ p53, ↑ p21 at 40 μg/mL of extract; ↑ Bax, ↓ Bcl-2, ↓ Bcl-xl, ↓ PI3K/Akt signaling pathways, at 10, 20 and 40 µg/ml extract, after 24h.	[231]

				In vivo: mice TGI (%)= 37, 47 and 61, at 10, 20, and 40 µg/mL of extract, respectively; TUNEL-positive cells (%): 6, 14 and 26 at 10, 20, 40 mg extract/kg	
S. officinalis (SO), S. fruticosa (SF), S. lavandulaefolia (SL)	Portugal	H_2O (RA, L-7-Glc)	Cytoprotection (Comet assay) and DNA repair	DNA protection against strand breaks induced by H_2O_2: 21% (HeLa cells, *SL*), 26% (Caco-2 cells, *SF*), at 50 µg/mL extract DNA repair in Caco-2 cells: 62% (*SO*), 86% (*SF*), at 50 µg/mL extract	[245]
S. miltiorrhiza leaves (L) and roots (R)	Commercial	EtOH, Acet (ND)	Cytotoxic effect (MTS), LDH release, amount of ATP and apoptosis on HepG2 and WRL-68 normal cells	MTS (IC_{50}, µg/mL)= HepG2: >200 (EtOH-L, Acet-L), 17 (EtOH-R), 83 (Acet-R), for 48h; WRL-68: 19->200 / 14->200, for 24/48h / LDH release: ↑ (EtOH- R) at 50 and 150 µg/mL for 18h / amount of ATP: ↓ (EtOH-R, Acet-R) / early apoptosis: ↑ (EtOH-L), late apoptosis/necrosis: ↑ (EtOH-R), after 100 µg/mL, for 24h	[238]
S. officinalis leaves (L) and roots (R)	Commercial	EtOH, Acet (ND)	Cytotoxic effect (MTS), LDH release, amount of ATP and apoptosis on HepG2 and WRL-68 normal cells	MTT (IC_{50}, µg/mL)= HepG2: 64 (EtOH-L), 90 (Acet-L), 20 (EtOH-R), 44 (Acet-R), for 48h; WRL-68: 100->120 / 46-88, for 24/48h / LDH release: ↑ (EtOH-L/R, Acet-L/R), at 50 and 150 µg/mL for 18h / amount of ATP: ↓ (EtOH-L/R, Acet-R) / early apoptosis: ↑ (EtOH-L, Acet-L), late apoptosis/necrosis: ↑ (EtOH-L/R), after 100 µg/mL, for 24h	[238]
S. officinalis	Algerian	EtOH-H_2O (RA, Flav)	Cytotoxic effect (MTT) on SK-N-BE(2)C neuronal, HepG2 hepatic cells	MTT (CVI %): 10 (SK-N-BE(2)-C), 25 (HepG2), for 48 h.	[176]
S. officinalis	Commercial	EtOH (RA, Salv K isomer, Lut-Hex, Api-Glr)	Cytotoxic effect (MTT), DNA damage (comet assay) and enzymatic activity of HepG2 cells	MTT (IC_{50}, mg/mL)= 5.7 / HepG2 DNA damage induced by H_2O_2 and DMNQ: ↓ after 2 mg/mL extracts, at 24h / Enzymatic activity: ↑ GPx, ↓ SOD, after 4, 2 and 1 mg/mL extracts	[178]
S. officinalis	Commercial	MeOH (ND)	Cytotoxic effect (MTT) on Raji, U937, KG-1A, MCF-7, PC3, WEHI-164 and in non-tumor HUVEC cells	MTT (IC_{50}, µg/mL)= Raji: 167; U937: 205; KG-1A: 179; MCF-7: 142; PC3: 76; WEHI-164: 40; HUVEC: > 600	[241]
S. officinalis	Commercial	EtOH, EtOH-H_2O, H_2O (ND)	Growth Inhibition (SRB), COX-2 inhibition (western blot) and cell cycle (flow cytometry) on HCA-7 cells	SRB (IC_{50}, µg/mL DW) = HCA-7: 347 (EtOH), 414 (EtOH-H_2O), > 442 (H_2O) / COX-2 inhibition (%): 40 (EtOH) at 40 µg GAE/ml; 70 (Celx) at 50 µM / HCA-7 cell cycle (%) = Sub G1: 16 (EtOH), 23 (Celx); G1: 42 (EtOH), 45 (Celx), S: 23 (EtOH), 18 (Celx), G2: 18 (EtOH), 13 (Celx), at 16 µg GAE/mL of EtOH and 50 µM of Celx	[169]

S. ringens	Macedonia	EtOH, H_2O (Kam-3-O-(6´´-O-acetil-Glu)-7-O-Rhm, rutin)	Cytotoxic effect (MTT) on HCT-116 and SW480 cells	MTT (IC_{50}, µg/mL) = HCT-116: 32 (EtOH), 9.8 (H_2O) at 24h / 179 (EtOH) / 407 (H_2O) at 72h / SW480: >500 (EtOH)/ >500 (H_2O) at 24h / >500 (EtOH), 412 (H_2O), at 72h	[114]
S. santolinifolia	Iran	DCM, MeOH, 80% MeOH (ND)	Cytotoxic effect (MTT) on HL60, K562, MCF-7 cells	MTT (IC_{50}, µg/mL)= HL60: 48 (DCM), 47 (MeOH), 49 (80% MeOH), 0.8 (Cis); K562: 79 (DCM), 49 (MeOH), 55 (80% MeOH), 2.1 (Cis) / MCF-7 Cells: 147 (DCM), 109 (MeOH), 6.9 (Cis)	[10]
Other *Salvia* species					
S. aegyptiaca, S. aethiopis, S. hypoleuca, S. limbata, S. nemorosa, S. syriaca, S. xanthocheila	Iran	DCM, MeOH, 80% MeOH (ND)	Cytotoxic effect (MTT) on HL60, K562, MCF-7	MTT (IC_{50}, µg/mL)= *S. aegyptiaca* - HL60: 99.7 (DCM); K562: 97 (DCM); MCF-7: 116 (DCM) / *S. aethiopis* - HL60: 45 (DCM); K562: 41 (DMC), 50 (MeOH); MCF-7: 44 (DMC), 79 (MeOH) / *S. hypoleuca* - HL60: 53 (DCM), 96 (MeOH); K562: 49 (DMC), 94 (MeOH); MCF-7: 83 (DMC), 106 (MeOH), 99 (80% MeOH) / *S. limbata* - HL60: 51 (DCM); K562: 45 (DMC), 111 (MeOH), 110 (80% MeOH); MCF-7: 64 (DMC), 149 (MeOH) / *S. nemorosa* - K562: 87 (80% MeOH) / *S. syriaca*- MCF-7: 75 (80% MeOH) / *S. xanthocheila* - HL60: 167 (MeOH); K562: 73 (MeOH); MCF-7: 128 (MeOH) / Cisplatin - HL60: 0.8; K562: 2.1; MCF-7: 6.9	[10]
Thymus species					
T. caramanicus	Iran	EtOH-H_2O	Cytotoxic effect (MTT) on MCF-7 cells / Biochemical markers of apoptosis and cell proliferation (Wester blot)	MTT (cell viability %) = MCF-7: 85 (EtOH-H_2O), 85 (Vin), 65(EtOH-H_2O+Vin) after 40 µg/mL extract; MCF-7: 70 (EtOH-H_2O), 85 (Vin), 50 (EtOH-H_2O+Vin), after 80 µg/mL extract / Wester blot = MCF-7 after 200 µg/mL extract: ↑ caspase 3, ↑ bax, ↓Bcl2, ↓ cyclin D1	[229]
T. schimperi	Ethiopia	MeOH 70% (Lut, Lut-7-O-Glc, Lut-7-O-xy)	Cytotoxic effect (MTT) on AGS and HepG2 cells	MTT (IC_{50}, µg/mL)= AGS: 88, after 50-100 µg/mL extract; HepG2: 326, after 200-400 µg/mL extract / MTT (cell inhibition %)= AGS: 38, HepG2: 35.	[147]
T. serpyllum (*Ts*), *T. vulgaris* (*Tv*)	Commercial	*Tv* H_2O (RA, CaffA, Lut-7-O-Glr, Lut-7-O-Rut, Erd-7-O-Rut, Ab) *Ts* H_2O (RA, CaffA, LAc, Lut-7-O-Glr, Lut-7-	Cytotoxic effect (MTT) on MCF-7/Adr or wt cells	MTT (IC_{50}, µg/mL)= MCF-7/Adr: 399 (*Ts*), 407 (*Tv*) / (IC_{50}, mM)= MCF-7/Adr: 0.81 (RA), 1.26 (LAc), 1.81 (CaffA), 1.87 (Lut-7-*O*-Glr), 4.2 (Lut-7-*O*-Rut), 2.6 (Erd-7-*O*-Rut), 5.8 (Ab); MCF-7/wt: 0.74 (RA), 1.09 (Lut-7-*O*-Glr), 0.45 (LAc), 1.36 (CaffA), 18.2 (Lut-7-*O*-Rut), 1.71 (Erd-7-*O*-Rut), >1000 (Ab) MTT (CVI %)= MCF-7/Adr: 60 (*Ts, Tv*), MCF-7/wt: 30 (*Ts*), 60 (*Tv*) at 500 mg/L extract / MCF-7/Adr: 86 (RA), 26 (Lut-7-*O*-Glr), MCF-7/wt: 92 (RA), 80 (LA), 54 (Lut-7-*O*-Glr), at 1.25 mM	[242]

		O-Rut, Erd-7-O-Rut)			
T. vulgaris	Commercial	EtOH (RA, Salv K isomer, Lut-Hex, Api-Glr)	Cytotoxic effect (MTT), DNA damage (comet assay); enzymatic activity of tumor HepG2 cells	MTT (IC_{50}, mg/mL)= 4.3 / HepG2 DNA damage induced by H_2O_2 and DMNQ: ↓ after 0.5 mg/mL extracts, at 24h / Enzymatic activity: ↑ GPx, ↓ SOD, after 1 and 0.5 mg/mL extracts	[178]
T. vulgaris	Commercial	PG (ND)	Cytotoxic effect (MTT, NR and CVA) on RAW 264.7, FMM-1, MCF-7 and HeLa cells	MTT (cell viability %)= RAW 264.7 and FMM-1: ↓ at 25, 50 and 100 mg/mL; MCF-7: ↓ at 50 and 100 mg/mL; HeLa cells: ↓ at 100 mg/mL extract NR (cell viability %)= RAW 264.7: ↓ at 50 and 100 mg/mL; FMM-1: ↓ no significant; MCF-7: ↓ at 25, 50 and 100 mg/mL; HeLa cells: ↓ at 50 and 100 mg/mL extract CVA (cell viability %): RAW 264.7: ↓ at 50 and 100 mg/mL; FMM-1: ↓ at 25, 50 and 100 mg/mL; MCF-7: ↓ at 100 mg/mL; HeLa cells: ↓ at 50 mg/mL extract	[208]
T. vulgaris	South Africa	Acet (ND)	Cytotoxic effect (XTT) on HeLa and non-tumor Vero cells	XTT (IC_{50}, μg/mL)= HeLa: > 200, 0.002 (AmD); Vero: 138, 0.027 (AmD)	[237]

A549 - human lung cancer cells; Acet – acetone; Adr -adriamycin-resistant; AGS - human gastric adenocarcinoma cells; AmD - actinomycin D; Ab - arbutin; BrdU– bromodeoxyuridine; Carn– carnosol; CarnA – carnosic acid; Celx – celecoxib; Cis - cisplatin; crytsh – cryptotanshinone; CVA - crystal violet assay; CVI - cell viability inhibition; DCM – dichloromethane; DMNQ - 2,3-dimethoxy-1,4-naphthoquinone; EA– ethyl acetate; Erd - eriodictiol; Flav – flavonoids; 5-Fl - 5-Fluorouracil; FMM-1 - human gingival fibroblasts; Glc - glucoside; Glc-82 - human lung cancer cells; Glr - glucuronide; GPx - glutathione peroxidase; H460 - human lung cancer cells; HCA-7 - colorectal cancer cells; MCF-7 - human breast cancer cells; HCT-116 colon carcinoma cells; HeLa - cervical carcinoma cells; HepG2 - hepatocellular carcinoma cells; HUVEC - human umbilical vein endothelial cells; KG-1A - human acute myelocytic leucemia cells; LAc - lithospermic acid; LDH – lactic dehydrogenase; Lut – luteolin, MeOH– methanol; MCF-7 - human breast carcinoma cells; MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt; MTT– 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ND– not determined; *n*-hex – *n*-hexan; NR - neutral red assay; OSCC - oral squamous cell carcinomas; PC3 - human prostate cancer cells; PG - Propylene glycol; RA– rosmarinic acid; Raji - non-Hodgkin's B-cell lymphoma; Rut - rutinoside; SOD - superoxide dismutase; SRB - Sulphorhodamine B assay; SW480 - colon adenocarcinoma cells; 6-OH-L-7-Glc – 6-hydroxyluteolin-7-glucoside, TGI - tumor growth inhibition rates; Tsh – tanshinone; TUNEL assay - terminal deoxynucleotidyl transferase dUTP nick end labeling; U937 - human leukemic monocyte lymphoma; Vin – vincristine; WEHI-164 - mouse fibrosarcoma cells; wt - wild-type; xy – xyloside; XTT - 2,3-bis-(2-methoxy-4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

1.3.4. Antibacterial activity

Infectious diseases involve complex interactions between the pathogens and the host. Pathogens enclose bacteria, fungus, virus or parasite, which can invade the host in different ways (e.g. respiratory, gastrointestinal, genitourinary tracts), when immune barriers are disrupted [188]. Overall, the infectious state is not only determined by the immunocompetence of the host, as well as the virulence of microorganisms, which is established by different factors contributing to the pathogenesis of infectious diseases [253].

The development of antibiotics has revolutionized the fight against infection by microbial agents, being able to control or even eradicate some infectious diseases in the world. However, the emergence and spread of resistance to these antibiotics in several microorganisms is difficulting the management of many infectious diseases [254]. Actually, the resistance to antibiotics has become a great concern to the World Health Organization (WHO), and antimicrobial resistance is recognized as an increasingly serious threat to global public health, being associated to diseases and high mortality rates, mainly at hospital level [254]. Thus, the search for new antimicrobial substances that might act as an alternative or complement existing antibiotics is imperative [20,47]. In this regard, purified natural compounds from plants can serve as one of the therapeutic strategies for the synthesis of new generation and alternative chemical drugs with low toxicity, mainly in antimicrobial and anti-inflammatory therapy [20,47,190].

Furthermore, foodborne diseases, that results from process of food production or from environmental contamination, encompass a wide spectrum of illnesses and also represent a a growing public health problem worldwide [255]. Moreover, a large variety of commercial antibiotics and food additives are used to control foodborne diseases, causing severe hypersensitivity reactions and increasing antibiotic resistance [255–257]. Thus, there it is important to reduce the rate of spoilage that impairs food quality, edibility and nutritional value, using suitable food preservation methods and resorting to organic food production [258]. The antibacterial activity of plant extracts and the presence of natural antioxidants can be useful as food preservatives because of their ability in controlling degradative reactions [2,20]. In the last years, the antimicrobial potential of essential oils from aromatic plants, especially from thyme and sage species, has been exploited with promising results [48,259–262]. Although less searched, the antimicrobial effect of polar extracts from *Salvia* and *Thymus* species has also been described, and in some cases recognized as more promising than essential oils [98,116].

Disc-diffusion and microdilution methods are the two most common methods for assessing antibacterial activity (Table 9). In the first assay, a disc is impregnated with drug/plant extracts,

and the effect is estimated by the measurement of inhibition zones (mm) around the disc [83,139]. In turn, in the microdilution assay, the microbial suspensions are prepared directly from the culture medium colonies and exposed to serial dilutions of antibiotic/plant extracts into wells of micro titer plates [263]. In these methods, the results can be delivered by the estimation of the minimum inhibitory concentration (MIC), that is defined as the lowest concentration at which visible growth is inhibit, as well as, the microbicidal (or bactericidal) concentrations (MMC/MBC), i.e., the lowest concentration of the tested substance which has a microbicidal effect (or bactericidal effect, if only bacteria is under research) [259,263]. MMC values are determined by sub-culturing from the MIC assay tubes onto appropriate culture medium and confirmation the lowest concentration with no visible growth [264].

Previous studies assessing antimicrobial activity of sage and thyme extracts (Table 9), focused the sensitivity of several microorganisms, namely Gram-negative and Gram-positive bacteria, mycobacteria and fungal strains. Note that emphasis is herein given to the antibacterial activity, since this topic will be addressed in this Thesis for the polar extracts of the target *Salvia* and *Thymus* species.

In general Gram-negative bacteria were shown to be more resistant than Gram-positive bacteria to plant extracts, mainly because of their high complex cell wall and the additional protection afforded by the outer membrane [265,266]. In particular, aqueous extracts of *S. ringens* [114] and *S. amplexicaulis* [106], both rich in kaempferol glycosides, inhibited the Gram-positive *Staphylococcus aureus* in lower concentrations than those required for Gram-negative specimens (*Escherichia coli* and *Pseudomonas aeruginosa*), when tested by the micro-well dilution assay [106]. Using the same method, a methanolic extract of *S. veneris* was claimed to have promising antimicrobial effects, particularly against *Listeria monocytogenes* (7.5 times higher than the antibiotic chloramphenicol), followed by *Proteus vulgaris, Bacillus cereus* and *Staphylococcus aureus* [98]. This effect was associated by the authors to rosmarinic acid and luteolin-*O*-glucoside, which are major phenolic components of *S. veneris*. Promising antibacterial results were also reported by Firuzi et al. [10] for different methanolic extracts from *Salvia* plants, including *S. eremophila, S. limbata, S. santolinifolia* and *S. sclarea,* which inhibited the growth of all the tested microorganisms at MIC values between 0.31-5.0 mg/mL.

In addition, based on both microdilution and disc-diffusion assays, a methanolic extract of *S. bicolor*, rich in gallic, *p*-coumaric and protocatchuic acids, was claimed to be ative against Gram-positive bacteria, especially *S. aureus* and *Staphylococcus epidermidis*, regardless it was 25-fold less active than gentamycin [139]. This extract also showed antibacterial effect towards Gram-negative bacteria, a fact that is related with agar disc diffusion results, whose larger halos (11.4 and 12.7 mm, for *P. aeruginosa* and *E. coli*, respectively) are in agreement

with the lowest MIC values (0.4 mg/mL) [139]. Moreover, considering both MIC and inhibition halos results, a methanolic extract of *S. nemorosa* (rich in rosmarinic acid, caffeic acid and quercetin) [73] and other of *Salvia spinosa* [267] were demonstrated to inhibit *S. aureus* in concentrations similar to gentamicin [73]. Besides, when testing the antibacterial effect of selected South African medicinal plants towards Gram-positive, Gram-negative and the mycobacteria *Mycobacterium tuberculosis*, Nielsen et al. [268] highlighted the promising effects of a methanolic extract of *S. africana-lutea*. This extract was able to prevent the growth of all the tested microorganisms (from which some were of clinical importance and most of them were found to be resistant to commonly used antibiotics), namely carbenicillin-resistant *P. aeruginosa* (CRPA), and the both β-lactamase positive (βL+) *E. coli* and methicillin-resistant *S. aureus* (MRSA), only 4-fold less active than gentamycin [268]. These authors showed also low MBC values for each drug-susceptible strains (MBC of 0.08 - 0.63 mg/mL) [268].

Table 9 summarizes diverse studies focusing the ability of *Salvia* polar extracts to inhibit fungal strains. Particularly, decoctions of *S. officinalis* were reported to provide high antifungal activity towards *Candida tropicalis* (inhibition halo greater than 11 mm), a fact that was positively related to their phenolic composition [180]. On another study, the growth of *Candida* species (e.g. *Candida albicans*) was shown to be inhibited by aqueous extracts of *S. amplexicaulis* [106] and methanolic extracts of *S. veneris* [98], *S. spinosa* [267], and *S. nemorosa* [73]. Notably, the antifungal ability of the last species was shown by the authors to be equal to that of the antifungic nystatin (MIC=0.05 mg/mL).

The antibacterial activity of *Thymus* extracts has also been the focus of attention by diverse authors [1,261]. In detail, Ertas et al. [83] reported that a methanolic extract of *T. nummularius*, which was rich in rosmarinic acid, luteolin and kaempferol, presented the same antibacterial activity than rosmarinic acid used as positive standard control [83]. Moreover, *Thymus lanceolatus* extracts of *n*-hexane, chloroform, ethanol and water, were shown to have high antimicrobial potential against a representative panel of infectious bacteria in (MIC values similar or even better than gentamicin) [269]. In addition, Ertürk et al. demonstrated that an ethanolic extract of *T. capitatus* was as active as the antibiotics amoxicilin and cefazolin against *S. aureus* (MIC=0.5 mg/ml) [270].

Despite some exceptions [83,208], similarly to sage, *Thymus* extracts have been recognized to be more effective against Gram-positive bacteria (e.g. *S. aureus* strains) than to Gram-negative specimens. In particular, an ethanolic extract of *T. capitatus* was reported to exhibit high antibacterial activity against *S. aureus* (MIC=0.5 mg/mL) comparatively to *P. aeruginosa* (MIC=2.0 mg/mL) [270]. Likewise, methanolic extracts of *T. capitatus* from Libya, were shown to have superior antibacterial effect against *S. aureus* (MIC of 5.0 mg/mL and inhibition zone of 13–18 mm) than to *Salmonella* species (MIC of 6.0 mg/mL and inhibition zone of 10–12 mm)

[271]. It should be noted for these *Thymus* extracts, MIC values taken from broth microdilution method were in accordance with the the diameter halos results gathered from the disc diffusion method. These evidences were also reported for different extracts of *T. lanceolatus* with a board-spectrum of activity [269]. In that study, the most sensitive strain was *S. aureus* (MIC=0.8 mg/ml), while the most resistant bacteria were the Gram-negative *Acinetobacter baumannii*, *Salmonella* species and *Enterobacter cloacae*, and the Gram-positive *L. monocytogenes* and *Enterococcus faecalis*(MIC values for all were equal to 4.0 mg/ml), which agree well with diameter halos by the disc diffusion method [269]. Distinct results were reported for hydroethanolic extracts of *T. vulgaris,* that presented the same inhibition magnitude against Gram-positive bacteria (including MRSA strains) and Gram-negative ones (*E. coli* and *P. aeruginosa* with MIC values of 6.25 mg/mL) [272]. Nevertheless, in that study, ethanolic extracts of *T. caramanicus* showed higher antibacterial efficacy than *T. vulgaris*, with MIC values comparatively below against the panel of tested strains [272].

The sensitivity of fungal strains, namely *Candida* strains, to thyme extracts was also tested before. In particular, ethanolic extracts of *T. capitatus* [270] and ethanolic extracts of *T. lanceolatus* [269] were demonstrated to inhibit strains of *C. albicans* (MIC of 1.0 and 0.1 mg/mL, respectively), with equal or more capacity than antifungal drugs nystatin and amphotericin B.

Despite, *Salvia* and *Thymus* extracts have been previously demonstrated to inhibit the growth of a wide spectrum of bacterial strains (Table 9), only little studies determined the MBC of the tested extracts, allowing the knowledge of the lowest concentration that has a microbicidal effect. In these sense, the knowledge of the inhibitory and lethal capacity of the tested extracts is important, according as, different concentrations have potential to inhibit the growth and/or the viability of bacteria strains under research. In addition, the findings described above suggest the possibility of using these extracts as food preservatives due to its inhibition of the growth of food spoilage and foodborne pathogens, as well as, in the development of new drugs for the treatment of infectious diseases. Taking together, these considerations underly the need of additional investigation regarding the antimicrobial potential of sage and thyme extracts.

Table 9 – Antimicrobial properties of phenolic-enriched extracts of distinct *Salvia* and *Thymus* species

Plant Species	Origin	Solvent extraction (major components)	Effect: screen assay	Results	Ref
Salvia species					
S. africana-lutea	South Africa	MeOH (ND)	Antibacterial and antifungal activity: broth microdilution method (MIC/MMC)	MIC (mg/mL)= Gram negative bacteria βL+EC: 0.04 (MeOH), 0.01 (Gen), ARKP: 0.31 (MeOH), 0.02 (Gen), CRPA: 0.16 (MeOH), 0.02 (Gen), CRCF: 0.31 (MeOH), 0.01 (Gen); Gram positive bacteria MRSA: 0.04 (MeOH), 0.01 (Gen); Mycobacteria *M. smegmatis*: 0.31 (MeOH), 0.0003 (Cpx), *M. tuberculosis*: 0.31 (MeOH), 0.00013 (Inz); Fungal strains *C. albicans*: 0.16 (MeOH), 0.02 (Nyt), *M. audouinii*: 0.04 (MeOH), 0.02 (Nyt) MMC (mg/mL)= Gram negative bacteria βL+EC: 0.08 (MeOH), 0.02 (Gen), ARKP: 0.63 (MeOH), 0.04 (Gen), CRPA: 0.31 (MeOH), 0.04 (Gen), CRCF: 0.63 (MeOH), 0.02 (Gen); Gram positive bacteria MRSA: 0.08 (MeOH), 0.02 (Gen); Mycobacteria *M. smegmatis*: 2.5 (MeOH), 0.0003 (Cpx); Fungal strains *C. albicans*: 0.31 (MeOH), 0.04 (Nyt), *M. audouinii*: 0.63 (MeOH), 0.04 (Nyt)	[268]
S. amplexicaulis	Macedonia	H_2O, EtOH (RA, Lut-5-O-Glc, Kam-3-O-(6´´-O-acetil-Glu)-7-O-Rhm)	Antibacterial and antifungal activity: Broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: 40 (EtOH), >50 (H_2O), 0.012 (Strm), *S. typhimurium* and *S. enteritidis:* 40 (EtOH), 50 (H_2O), 0.010 (Strm), *P. tolasii*: 35 (EtOH), 50 (H_2O), 0.016 (Strm), *P. aeruginosa*: 40 (EtOH), >50 (H_2O), 0.016 (Strm), *P. mirabilis*: 35 (EtOH), >40 (H_2O), 0.005 (Strm); Gram positive bacteria *S. aureus:* 30 (EtOH), 40 (H_2O), 0.016 (Strm), *B. cereus*: 30 (EtOH), 35 (H_2O), 0.005 (Strm), *M. flavus*: 30 (EtOH), 35 (H_2O), 0.010 (Strm), *S. lutea*: 40 (EtOH), >40 (H_2O) 0.012 (Strm), *L. monocytogenes*: 30 (EtOH), 40 (H_2O), 0.010 (Strm); Fungal strains *C. krusei*: 64 (H_2O), *C. albicans*: 32 (H_2O), *C. parapsilosis*: 16 (H_2O), 0.0078 (ktz)	[106]
S. bicolor	Egypt	MeOH (PrcA, CouA, GA, Lut-7-O-Glc)	Antibacterial and antifungal activity: agar disc diffusion / broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: 0.4 (MeOH), $8x10^{-3}$ (Gen), *P. aeruginosa* and *S. boydii:* 0.4 (MeOH), 0.01 (Gen), *K. pneumonia* and *P. vulgaris:* 1 (MeOH), 0.01 (Gen); Gram positive bacteria *S. aureus:* 0.2 (MeOH), $8x10^{-3}$ (Gen), *S. epidermidis*: 0.35 (MeOH), 0.01 (Gen), *S. pyogens*: 0.35 (MeOH), $8x10^{-3}$ (Gen); Fungal strains *C. albicans*: 0.35 (MeOH), $1x10^{-3}$ (Amb), *C. krusei*, *C. glabrata. and C. parapsilosis:* 1 (MeOH), $1x10^{-3}$ (Amb)	[128]

S. nemorosa	Iran	MeOH (RA, CaffA, Que)	Antibacterial and antifungal activity: agar disc diffusion / broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: >1 (*n*-Hex), 0.8 (DCM), 0.1 (MeOH), 0.025 (Gen), *P. aeruginosa:* >1 (*n*-Hex), 0.8 (DCM), 0.4 (MeOH), 0.05 (Gen); Gram positive bacteria *S. aureus:* 0.1 (*n*-Hex), 0.01 (DCM), 0.01 (MeOH), 0.01 (Gen), *B. cereus:* 0.1 (*n*-Hex), 0.1 (DCM), 0.05 (MeOH), 0.01 (Gen); Fungal strains *C. albicans*: 1 (*n*-Hex), 1 (DCM), 0.2 (MeOH), 0.05 (Nyt), *A. niger*: 1 (*n*-Hex), 0.2 (DCM), 0.05 (MeOH), 0.05 (Nyt)	[73]
S. ringens	Macedonia	H_2O, EtOH (CaffA, Kam-3-O-(6´´-O-acetil-Glu)-7-O-Rhm, rutin)	Antibacterial and antifungal activity: broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: 15 (EtOH), 25 (H_2O), 0.012 (Strm), *S. typhimurium:* 15 (EtOH), 25 (H_2O), 0.012 (Strm), *S. enteritidis:* 15 (EtOH), 20 (H_2O), 0.010 (Strm), *P. tolasii* and *P. aeruginosa*: 20 (EtOH), 30 (H_2O), 0.016 (Strm), *P. mirabilis*: 35 (EtOH), >40 (H_2O), 0.005 (Strm); Gram positive bacteria *S. aureus:* 5 (EtOH), 15 (H_2O), 0.016 (Strm), *B. cereus*: 10 (EtOH), 20 (H_2O), 0.005 (Strm), *M. flavus*: 10 (EtOH), 20 (H_2O), 0.010 (Strm), *L. monocytogenes*: 5 (EtOH), 15 (H_2O), 0.010 (Strm), *S. lutea*: 15 (EtOH), 25 (H_2O), 0.012 (Strm); Fungal strains *C. krusei*: 64 (EtOH), *C. albicans*: 64 (EtOH), 0.0078 (ktz)	[114]
S. spinosa	Iran	*n*-Hex, DCM, MeOH (ND)	Antibacterial and antifungal activity: agar disc diffusion / broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: >1 (*n*-Hex), >1 (DCM), 0.2 (MeOH), 0.025 (Gen), *P. aeruginosa:* >1 (*n*-Hex), >1 (DCM), 0.8 (MeOH), 0.05 (Gen); Gram positive bacteria *S. aureus:* 0.01 (*n*-Hex), 0.05 (DCM), 0.01 (MeOH), 0.01 (Gen), *B. cereus:* 0.05 (*n*-Hex), 0.1 (DCM), 0.01 (MeOH), 0.01 (Gen); Fungal strains *C. albicans*: 0.2 (*n*-Hex), 0.8 (DCM), 0.05 (MeOH), 0.05 (Nyt), *A. niger*: 0.8 (*n*-Hex), 0.4 (DCM), 0.1 (MeOH), 0.05 (Nyt)	[267]
S. veneris	Cyprus	MeOH (RA, Lut-Glc)	Antibacterial and antifungal activity: broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *P. aeruginosa*: 0.5 (MeOH), 0.008 (Clp), E. *aerogenes:* 2 (MeOH), 0.002 (Clp), *P. vulgaris*: 0.25 (MeOH), 0.004 (Clp), E. *coli* and *S. marcescens*: 2 (MeOH), 0.004 (Clp), *E. coli* O157:H7: 1 (MeOH), 0.002 (Clp), *S. typhimurium*: 2 (MeOH), 0.032 (Clp); Gram positive bacteria *S. aureus*: 0.25 (MeOH), 0.002 (Clp), *S. epidermidis*: 2 (MeOH), 0.002 (Clp), *L. monocytogenes* 0.06 (MeOH), 0.008 (Clp), *B. cereus*: 0.25 (MeOH), 0.004 (Clp), *B. subtilis*: 0.5 (MeOH), 0.004 (Clp); Fungal strains *C. albicans*: 0.5 (MeOH), 0.0005 (Amb), *C. utilis*: 0.06 (MeOH), 0.00025 (Amb), *C. krusei*: 0.25 (MeOH), 0.0005 (Amb), *C. glabrata* and *C. tropicalis*: 0.500 (MeOH), 0.0001 (Amb), *C. parapsilosis:* 0.12 (MeOH), 0.0005 (Amb)	[98]
S. eremophila (Se), S. limbata (Sl), S. santolinifolia (Ssa), S. sclarea (Ssc)	Iran	80% MeOH (ND)	Antibacterial ativity: agar disc diffusion / broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: 2.5 (*Se, Ssa*), 5 (*Sl, Ssc*), 0.05 (Clp), *K. pneumonia*: 2.5 (*Se, Sl, Ssc*), 5 (*Ssa*), 0.05 (Clp), *S. typhimurium*: 0.31 (*Se, Ssc*), 1.25 (*Sl, Ssa*), 0.05 (Clp),; Gram positive bacteria *B. subtilis*: 2.5 (*Se, Ssa*), 5 (Sl, *Ssc*), 0.0125 (Clp), *S. epidermidis*: 0.3 (*Se, Ssa*), 1.25 (*Ssa*), 2.5 (*Sl*), 0.025 (Clp), *S. aureus*: 0.6 (*Se*), 1.25 (*Ssc*), 2.5 (*Sl, Ssa*), 0.0125 (Clp)	[10]

Thymus species					
T. capitatus	Turkey	EtOH (ND)	Antibacterial and antifungal activity: agar disc diffusion method / agar dilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: 8 (EtOH), ≤ 0.5 (Amx, Cfz), *P. aeruginosa:* 2 (EtOH), ≤ 0.5 (Amx, Cfz); Gram positive bacteria *S. aureus:* 0.5 (EtOH), ≤ 0.5 (Amx, Cfz), *B. subtilis:* 4 (EtOH), ≤ 0.5 (Amx, Cfz); Fungal strains *C. albicans*: 1 (EtOH), ≤ 2 (Nyt), *A. niger*: 0.5 (EtOH), ≤ 2 (Nyt)	[270]
T. capitatus	Libya	MeOH (ND)	Antibacterial activity: agar disc diffusion method / agar dilution method (MIC)	MIC (mg/mL)= *S. aureus:* 5; *B. subtilis:* 1.25; *Salmonella* species: 6	[271]
T. lanceolatus	Algeria	Hex, Chl, EtOH, H_2O (ND)	Antibacterial and antifungal activity: agar disc diffusion method / broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *A. baumannii*: 4 (Hex), 0.125 (Chl), 0.125 (EtOH), 4 (H_2O), 2 (Gen); *C. freundii*: 2 (Hex), 1 (Chl), 0.5 (EtOH), 2.7 (Gen); *E. coli*: 4 (Hex), 1 (Chl), 0.5 (EtOH), 2 (gen); *E. cloacae*: 1 (Chl), 0.25 (EtOH), 4 (H_2O), 2 (Gen); *K. pneumonia*: 1 (Chl), 0.5 (EtOH), 1.3 (Gen); *P. mirabilis*: 1 (Chl), 0.21 (EtOH), 2 (H_2O), >8 (Gen); *P. aeruginosa:* 2 (Chl), 0.25 (EtOH), 1 (Gen); *P. fluorescens*: 1 (Chl), 0.25 (EtOH), 1 (Gen); *S. enteritidis:* 1.33 (Chl), 0.5 (EtOH), 4 (H_2O), 2 (Gen); *S. typhimurium*: 4 (Hex), 0.5 (Chl), 0.25 (EtOH), 4 (H_2O), 2 (Gen); Gram positive bacteria *B. cereus*: 0.08 (Chl), 0.125 (EtOH), 4 (Gen); *B. subtilis*: 0.125 (Chl), 0.25 (EtOH), 4 (Gen); *E. faecalis*: 2 (Hex), 0.03 (Chl), 0.1 (EtOH), 4 (H_2O), 8 (Gen); *L. monocytogenes*: 0.03 (Chl), 0.25 (EtOH), 4 (H_2O), 2 (Gen); *S. aureus*: 0.03 (Chl), 0.06 (EtOH), 0.8 (H_2O), 0.25 (Gen); Fungal strains *C. albicans*: 0.125 (Chl), 0.1 (EtOH), 1 (Amb)	[269]
T. nummularius	Turkey	MeOH (QA, RA, Lut, Kam)	Antibacterial activity: Agar disc diffusion method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: > 1 (MeOH, RA), *P. aeruginosa*: > 1 (MeOH, RA); Gram positive bacteria *S. aureus*: > 1 (MeOH, RA), *S. pyogenes*: > 1 (MeOH, RA)	[83]
T. vulgaris	Commercial	PG (ND)	Antibacterial and antifungal activity: broth microdilution method (MIC	MIC / MMC (mg/mL)= *C. albicans*: 50 /50; *S. aureus, E. faecalis, S. mutans and P. aeruginosa:* > 50 / > 50 / biofilms reductions in CFU/mL after exposure to *T. vulgaris* L. extract (200 mg/mL)	[208]

			/MMC) / biofilms reduction (CFU/ml)		
T. vulgaris (Tv), T. caramanicus (Tcar)	Iran	EtOH-H_2O (ND)	Antibacterial activity: agar disc diffusion method / broth microdilution method (MIC)	MIC (mg/mL)= Gram negative bacteria *E. coli*: 6.25 (*Tv*), 1.56 (*Tcar*), *P. aeruginosa:* 6.25 (*Tv*), 1.30 (*Tcar*); *S. dysenteriae*: 12.5 (*Tv*), 0.78 (*Tcar*); *S. typhimurium*: 12.5 (*Tv*), 2.6 (*Tcar*); Gram positive bacteria *S. aureus:* 6.25 (*Tv*), 0.78 (*Tcar*); *S. epidermidis*: 6.25 (*Tv*), 1.04 (*Tcar*); *B. subtilis:* 10.4 (*Tv*), 1.56 (*Tcar*); MRSA: 6.25 (*Tv*), 1.30 (*Tcar*)	[272]

Amb - amphotericin-B; Amx – amoxicillin; CaffA – caffeic acid; CFU - colony-forming unit; Cfz – cefazolin; Chl - chloroform; Clp - chloramphenicol; CouA - coumaric acid; Cpx – ciprofloxacin; DCM– dichloromethane; EA– ethyl acetate; GA – gallic acid; Gen - gentamicin; Glc - glucoside; Hex – hexane; Inz – isoniazid; Kam – kaempferol; Ktz - ketoconazole; Lut – luteolin; MeOH– methanol; MIC - minimum inhibitory concentrations; MMC - minimum microbicidal concentration; ND– not determined; Nyt – nystatin; PG - propylene glycol; PrcA - protocatchuic acid; QA - quinic acid; Que – quercetin; RA– rosmarinic acid; Rhm - rhamnoside; Strm – streptomycin.

Microbial names: *A. baumannii - Acinetobacter baumannii*; *A. niger - Aspergillus niger*, ARKP - ampicillin-resistant *Klebsiella pneumonia*; *B. subtilis - Bacillus subtilis*; *C. albicans - Candida albicans, C. freundii- Citrobacter freundii; C. krusei - Candida krusei; C. glabrata - Candida glabrata,; C. parapsilosis - Candida parapsilosis;* CRPA - carbenicillin-resistant *Pseudomonas aeruginosa;* CRCF- chloramphenicol-resistant *Citrobacter; C. tropicalis - Candida tropicalis; C. utilis - Candida utilis;* βL+EC - β-lactamase positive *Escherichia coli; B. cereus - Bacillus cereus; B. subtilis - Bacillus subtilis; E. aerogenes - Enterobacter aerogenes; E. cloacae - Enterobacter cloacae; E. coli - Escherichia coli*; *E. faecalis - Enterococcus faecalis; K. pneumoniae - Klebsiella pneumoniae*; *L. monocytogenes - Listeria monocytogenes*; *M. audouinii - Microsporum audouinii*; *M. flavus - Micrococcus flavus*; MRSA - methicillin-resistant *Staphylococcus aureus*; *M. smegmatis - Mycobacterium smegmatis*; *M. tuberculosis - Mycobacterium tuberculosis*; *P. aeruginosa - Pseudomonas aeruginosa*; *P. fluorescens - Pseudomonas fluorescens*; *P. mirabilis - Proteus mirabilis*; *P. vulgaris - Proteus vulgaris; P. tolasii - Pseudomonas tolasii*; *S. aureus - Staphylococcus aureus; S. boydii - Shigella boydii; S. dysenteriae - Shigella dysenteriae; S. epidermidis - Staphylococcus epidermidis*; *S. lutea -Sarcina lutea, S. enteritidis - Salmonella enteritidis; S. dysenteriae - Shigella dysenteriae; S. marcescens - Serratia marcescens, S. pyogenes - Streptococcus pyogenes¸ S. typhimurium - Salmonella typhimurium.*

1.3.5. Effects of *Salvia* and *Thymus* plants in diabetes and obesity

The health-benefits of phenolic compounds, especially flavonoids and phenolic acids, have been associated to the risk control of metabolic syndrome and related complications such as obesity, diabetes and associated disorders (e.g. cardiovascular disease, neuropathy, nephropathy and retinopathy) [47,160]. In particular, these compounds are claimed to inhibit the activity of α-glucosidase, α-amylase and pancreatic lipase, which represent key digestive enzymes involved in the metabolism of carbohydrates and lipids. α-Amylase and α-glucosidase are responsible for the digestion of dietary carbohydrates to glucose, which enters in the blood stream. Thus, inhibiting the activity of these enzymes results in the slowing of liberation and absorption of glucose, which in turn, reduce blood glucose level and might contribute to the suppress of hyperglycemia and diabetes [160,273,274]. In turn, lipase inhibition decreases the digestion of dietary triglycerides in the small intestine [275]. In addition, dietary plant polyphenols are effective against body weight gain (bw), fat deposition and adipocyte dysfunction. Thus, these compounds modulates carbohydrate and lipid metabolism, attenuate hyperglycemia, dyslipidemia and insulin resistance, and weaken the oxidative stress and related inflammatory processes [160,273–275].

Since both acute and chronic inflammatory responses are associated with the development of diabetes and insulin resistance (and these conditions are commonly observed in obesity), plant-based polyphenolic compounds represent important contributors in prevention of metabolic disorder associated with hyperglycemia and diabetes [160]. In these sense, in some countries, various anti-inflammatory and antioxidant plants have been used traditionally as an alternative strategy for the treatment of diabetes [1,276].

Researches focusing the potentialities of polar extracts of *Salvia* and *Thymus* plants to act as natural antidiabetic and antiobesity agents, have mainly tested their ability to control glucose levels through inhibition of α-glucosidase activity. In particular, the ability of *Salvia* plants in inhibiting this enzyme was reported for *S. officinalis* (IC_{50} value of 69.7 μg/mL), which was shown to be much stronger than acarbose (IC_{50}=203 μg/mL) [277]. The α-glucosidase inhibition was also demosntrated for methanolic extracts of *Salvia acetabulosa*, *S. nemorosa* and *S. chloroleuca* (IC_5 =76.9 μg/mL, IC_{50}=19 μg/mL and IC_{50}=13.3 μg/mL, respectively) [73,278,279]. Likewise, methanolic extracts of *T. quinquecostatus* were shown to have an inhibitory effect on α-glucosidase activity (IC_{50}=4.39 μg/ml) [280]. The inhibitory capacity of polar extracts from *Salvia* and *Thymus* plants towards α-amylase and/or α-glucosidase have been also mostly correlated with their phenolic constituents. In fact, aqueous and methanolic extracts of *S. cadmica*, both

rich in rosmarinic acid, luteolin and apigenin, had high inhibitory effects against these two enzymes [71]. Moreover, several phenolic compounds isolated from *S. miltiorrhiza*, namely, tanshinone IIA, rosmarinic acid, rosmarinic acid methyl ester and salvianolic acid C methyl ester, were reported to be stronger inhibitors of α-glucosidase than acarbose [281]. Likewise, the flavonoid compounds luteolin-7-*O*-glucoside, luteolin-7-*O*-glucuronide and diosmetin-7-*O*-glucuronide, isolated from the aerial parts of *S. chloroleuca*, also showed potent α-glucosidase inhibitory effects (IC_{50} of 18.3, 14.7, and 17.1 μM, respectively), exhibiting an inhibitory effect close to that of acarbose (IC_{50}=16.1 μM) [279]. The inhibition of lipase activity by polar extracts of *Salvia* origin was previously reported in particular for the methanolic extracts of *S. officinalis* leaves [282], *S. spinosa* [283] and *Salvia triloba* [284], which reported IC_{50} values of 94.0, 156.2 and 100.8 μg/mL, respectively.

Notably, the antidiabetic ability of sage and thyme extracts has also been demonstrated in *in vivo* models. A methanolic extract of *S. officinalis* (500 mg/kg bw) was shown to control the activity of α-glucosidase, alike acarbose (20 mg/kg bw) in alloxan-treated diabetic rats [285]. Moreover, Cam et al. [274] demonstrated the antidiabetic effects of methanolic extract of *Thymus praecox* subsp. *skorpilii* var. *skorpilii* in streptozotocin/nicotinamide-induced type 2 diabetic rats by restoring glucose homeostasis, ameliorating insulin resistance and improving pancreatic β-cell function [274]. The authors ascribed these positive effects to the phenolic composition of the extract (chlorogenic acid, luteolin-7-*O*-glucoside, 3-*O*-feruloylquinic acid, quercetin-3-*O*-hexoside and apigenin-7-*O*-glucuronide) [274]. In addition, the treatment of diabetic rats with hydroalcoholic extracts of *T. caramanicus* (300 and 500 mg/kg bw) caused a significant reduction on the blood glucose level, serum total cholesterol, triglycerides, creatinine and liver function enzymes. In addition, it inhibited α-amylase and the reabsorption of glucose through the decrement of renal glucose transporters gene expressions [286].

In addition, clinical trials have evaluated the efficacy and safety of *S. officinalis* L. leaf extracts or tablets in the treatment of patients with hyperlipidemia and type-2 diabetes, for which it was shown to cause a decrease on blood levels of triglyceride, total cholesterol, low density lipoproteins (LDL) postprandial glucose after 2 h, and to increase the levels of high density lipoprotein (HDL) [174,287–289]. In another study, Sá et al. [290] reported the ability of a sage tea drinking in improving lipid profile, decreasing the plasma total and LDL cholesterol levels and increasing plasma HDL cholesterol levels during and two weeks after treatment. The authors evidenced also that sage tea drinking improved human erythrocyte antioxidant status by significantly increasing SOD and CAT activities, thus ameliorating antioxidant defenses, which may indirectly improve the

diabetic condition. Importantly, some of these studies indicate the safety of *S. officinalis* since it did not cause hepatotoxicity nor induce other adverse effects, such as changes in blood pressure, heart rate at rest and body weight [287,288,290].

1.3.6. Future considerations

It is well know that diet supplementation with plant-based products, which contain large amount of polyphenols and phytochemicals [2,160], can provide positive influence on human health, reducing the risk of cancer, diabetes and inflammatory diseases, and preventing neurodegenerative and cardiovascular system disorders [14,235,236]. The bioactivity of plant secondary metabolites that are still unexplored, enable them to development of functional foods, nutraceuticals and drugs based on plants, that could be the future of health supplies for world population [14,291]. In addition, antibacterial and antioxidant properties of plants and their secondary metabolites could be used in the development of new agents, with application in pharmaceutical and food industries, as drugs metabolites against infectious diseases or as preservative additives [2,20,292].

There is a clear trend in consumer preference towards food ingredients and additives that are natural and that are perceived to be healthy [293]. In these sense, being thyme and sage plants rich in phenolic acids and flavonoids, with health-beneficial activities protecting against inflammatory chronic diseases such as diabetes, cancers, ageing and others [1,47,158,159], their extracts have also been proposed to have promising applicability for the development of safe products and additives [1,65,294].

In recent years, a number of innovative therapeutic strategies are being developed in order to combat inflammatory associated diseases, namely glucocorticoids-nanoformulations (e.g liposomes, nanoparticles, and organic and inorganic scaffolds) [295,296]. Based on the knowledge of plants as a source of various biologically active compounds, recent researches were based in production of biocompatible and environmentally safe nanoparticles of plant origin for medical applications. Furthermore, multiple approaches are needed to surpass limited solubility of phenolic compounds, including the development of novel drug delivery system and formulations such as nanoparticles, liposomal encapsulation, emulsions, and sustained released tablets [193,273,297]. Therefore, health-benefits promising-results and enhanced bioavailability of phenolic compounds from thyme and sage plants, could bring these promising natural products to the therapeutic agents for several diseases.

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3. RESEARCH AIM

Salvia and *Thymus* plants are endowed of biochemical and organoleptic properties that enable their application in distinct fields such as culinary, cosmetic or medicinal. Importantly, their usage may be done in different procedures, that range from the direct application of fresh or dry plants, to essential oils and extracts, which can be obtained in different forms, including aqueous infusions and/or decoctions.

Importantly, even though for the last years there has been an increasing number of researches aiming to screen of the biological properties of *Salvia* and *Thymus* plants, many species and/or cultivars remains poorly studied. Based on known bioactivities, as well as insufficient data of health properties of several less-known sage and thyme species, this study was aimed to provide comprehensive research of chemical composition and biological effects of aqueous extracts of several *Salvia* and *Thymus* species, with potential applications in food, pharmaceutical and cosmetic industries.

In this context, the present Doctoral Thesis is intended to elucidate the phenolic composition and the beneficial properties of eigth *Salvia* (*S. officinalis*, *S. elegans*, *S. officinalis 'Icterina'*, *S. apiana*, *S. farinacea* var. *victoria blue*, *S. africana*, *S. greggii*, and *S. mexicana*) decoctions and of six *Thymus* (*T. herba barona, T. pseudolanuginosus, T. caespititius, T. zygis, T. fragrantissimus* and *T. pulegioides*) decoctions. Specific aims were defined as:

First aim: Establish the phenolic profile of *S. officinalis*, *S. elegans*, *S. officinalis 'Icterina'*, *S. apiana*, *S. farinacea* var. *victoria blue*, *S. africana*, *S. greggii*, and *S. mexicana*, *T. herba-barona, T. pseudolanuginosus, T. caespititius, T. zygis, T. fragrantissimus* and *T. pulegioides* decoctions by ultra-high performance liquid chromatography coupled to diode array detector and an electrospray mass spectrometer (UHPLC-DAD-ESI-MS^n).

Second aim: Determine the antioxidant potential of decoctions from the target sage and thyme plants using distinct chemical methods (scavenging of $DPPH^•$, $NO^•$ and $O_2^{•-}$; ferric reducing power, TBARS inhibition, β-carotene bleaching, xanthine oxidase inhibition activity and ORAC assays.

Third aim: Evaluate the capacity of decoctions of *T. herba barona, T. pseudolanuginosus, T. caespititius, S. officinalis 'Icterina'*, *S. apiana*, *S. farinacea* var. *victoria blue*, *S. africana*, and *S. mexicana* to counteract inflammatory events, namely by reducing the levels of nitric oxide, as assessed in chemical and/or in cellular models.

Fourth aim: Evaluate the antibacterial activity of six *Thymus* decoctions (*T. herba barona, T. pseudolanuginosus, T. caespititius, T. zygis, T. fragrantissimus* and *T. pulegioides*), and of five *Salvia* (*S. apiana, S. farinacea* var. *victoria blue, S. africana, S.*

mexicana and *S. officinalis 'Icterina'*) decoctions against Gram-positive (*Staphylococcus epidermidis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa*).

Fifth aim: Evaluate the cytotoxic potential of decoctions of *S. apiana, S. farinacea* var. *victoria blue, S. africana, S. mexicana* and *S. officinalis 'Icterina'*, towards four cancer cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma).

Sixth aim: Evaluate the inhibitory ability of *S. elegans* and *S. greggii* decoctions against key enzymes with impact in obesity and diabetes (α-glucosidase, α-amylase and pancreatic lipase), comparing the findings to those of the well-known *S. officinalis* species.

Seventh aim: Evaluate the nutritional profile of *T. zygis, T. fragrantissimus* and *T. pulegioides*, in particular total carbohydrates, proteins, ach, fatty acids and minerals (Na, K, Ca, Mg, Fe, Mn, Cu, and Zn).

4. ORIGINAL PUBLICATIONS

Beneficios en la salud de extractos aquosos de *Thymus herba-barona, Thymus pseudolanuginosus* y *Thymus caespititius*

Objetivos

Caracterizar y cuantificar los componentes fenólicos y evaluar las actividades antioxidante, antiinflamatoria y antibacteriana de extractos acuosos de tres especies de tomillo - *Thymus herba-barona, Thymus pseudolanuginosus* y *Thymus caespititius*.

1. Introducción

El género *Thymus* incluye alrededor de 350 especies, que están particularmente bien adaptadas al clima cálido y seco de la región mediterránea y se extienden en las zonas áridas de la Península Ibérica [1,2]. En cuanto a la gran mayoría de las plantas Lamiaceae, se reconoce que el tomillo es muy aromático y se usa ampliamente para mejorar el sabor y la conservación de los alimentos [1]. Asimismo, estas plantas tienen cierto potencial en las industrias farmacéutica y cosmética debido a sus beneficios biológicos y medicinales [3–6].

Varios autores han demostrado que plantas pertenecientes a la familia Lamiaceae, y particularmente las plantas de tomillo, son ricas en fitoquímicos bioactivos, incluidos los ácidos fenólicos y flavonoides [1,7–9], que a su vez se han descrito como agentes capaces de reducir el riesgo de enfermedades debido a sus extensas propiedades biológicas, incluyendo antioxidante, cardioprotector, antitumorales, antienvejecimiento, antiinflamatorio y antibacteriano [4,5,10,11]. La evaluación de los posibles efectos bioactivos de los extractos polares de *Thymus* se realizó en estudios previos principalmente para *Thymus vulgaris* [12–15], *Thymus serpyllum* (tomillo silvestre) [2,16], y en algunas otras especies de tomillo. En general, se ha demostrado que los extractos acuosos o alcohólicos de *Thymus* exhiben fuertes actividades antioxidantes *in vitro* [17–19] y en modelos *in vivo* [17–19]. Además, otros efectos descritos en los extractos polares de especies de tomillo incluyen antimicrobiano [20,21], antidiabético [11,17], neuroprotector [15] y propiedades antiinflamatorias [22].

En contraste con los estudios mencionados anteriormente, las aplicaciones potenciales de los extractos acuosos de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius* permanecen sin explorar. El presente estudio tiene como objetivo contribuir a la clarificación de los constituyentes fenólicos de estas tres especies de tomillo, así como explorar sus actividades antioxidantes, antibacterianas y antiinflamatorias.

2. Métodos

2.1. Plantas

Las plantas *T. herba-barona, T. pseudolanuginosus y T. caespititius*, compradas a la empresa Ervital (Viseu, Portugal), se cultivaron bajo un régimen orgánico y, después de la recolección, sus partes aéreas (flores, hojas y tallos) se secaron en una incubadora ventilada a 20-35ºC, durante 3 a 5 días.

2.2. Extracción de compuestos fenólicos

Los extractos fenólicos de los tres tomillos se han obtenido utilizando una decocción en la proporción 1:20 (5 g en 100 ml de agua). Las soluciones se filtraron, se concentraron y el concentrado se desengrasó con *n*-hexano (1:1 *v/v*), se evaporó y se congeló a -20°C y se liofilizó. Se obtuvieron tres extractos de cada planta.

2.3 Identificación y cuantificación de compuestos fenólicos

El contenido fenólico total de cada extracto de tomillo se determinó de acuerdo con el método colorimétrico de Folin-Ciocalteu, cuyos valores se expresaron en equivalentes de ácido gálico (EAG)/g de extracto o mg EAG/g de planta.

Los extractos resultantes fueron caracterizados y cuantificados por cromatografía líquida de alta eficiencia (UHPLC), asociada a espectrometría de masas por electrospray (ESI-MS) en el modo de ionización negativo y MS^n.

Para el análisis cuantitativo, los límites de detección (LOD) y cuantificación (LOQ) se calcularon a partir de los parámetros de las curvas de calibración obtenidas mediante la inyección de concentraciones conocidas de los compuestos estándar exactos o relacionados estructuralmente.

2.4 Pruebas de bioactividad

2.4.1 Capacidad antioxidante

La actividad antioxidante de los extractos acuosos de tomillo se evaluó mediante ensayos químicos, utilizando la prueba de radicales 2,2-difenil-2-picrilhidrazilo ($DPPH^\bullet$), el cálculo del poder reductor por la reducción de hierro (III) y el blanqueo del β-caroteno. En la primera prueba se utilizó ácido ascórbico como control positivo y en los otros dos métodos se utilizó hidroxianisol butilado (BHA) como estándar. Los resultados se expresaron como valores de EC_{50} (concentración de la muestra que proporciona el 50% de la actividad antioxidante) en µg por ml de extracto.

2.4.2 Capacidad antiinflamatoria

Las propiedades antiinflamatorias de los tres extractos acuosos de tomillo se evaluaron *in vitro* mediante modelos químicos, determinándose las cantidades de óxido nítrico ($NO^\bullet$) producido y el efecto inhibidor del extracto frente a la actividad de la enzima 5-lipoxigenasa (5-LOX). Se utilizó ácido ascórbico como control positivo. Los resultados

se expresaron en valores de EC_{50} (µg/ml), que corresponden a la concentración de la muestra que proporcionó el 50% de inhibición de la producción de NO o 5-LOX.

2.4.3 Capacidad antibacteriana

El potencial antibacteriano de los extractos polares de *Thymus* se evaluó frente a cinco cepas bacterianas, incluyendo dos Gram positivas (Gram+) *Staphylococcus epidermidis* y *Staphylococcus aureus*, y tres Gram negativas (Gram-) *Salmonella typhimurium, Escherichia coli* y *Pseudomonas aeruginosa*, por el método de microdilución en medio, lo que permitió el cálculo de la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM). Se utilizó nisina como control positivo.

3. Resultados y discusión

3.1. Compuestos fenólicos en extractos acuosos de *Thymus*

El rendimiento de extracción de los extractos acuosos de tomillo osciló entre 15 y 20%, siendo *T. caespetitius* el asociado a mayores rendimientos (Tabla 1). A su vez, este último mostró un contenido en fenólicos totales más bajo (236.0 mg EAG/g de extracto, equivalente a 47.0 mg EAG/g de planta seca), en comparación con los valores medios obtenidos en los extractos de *T. herba-barona* y *T. pseudolanuginosus* (272.9 y 293.0 mg EAG/g de extracto o 41.6 y 49.2 mg EAG /g de planta seca), respectivamente.

Tabla 1. Rendimiento de extracción (%), compuestos fenólicos totales (mg EAG/g de extracto), actividad antioxidante y antiinflamatoria (EC_{50}, µg/mL) de extractos acuosos de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius*

Extracto	Rendimiento (%)	CFT	DPPH•	PR	β-Caroteno	NO•	5-LOX
T. h-b	15.3 ± 1.8^{a}	272.9 ± 16.6^{ac}	11.6 ± 0.9^{a}	35.1± 4.5^{a}	> 26.7^{a}	286.1 ± 32.6^{a}	840.8 ± 137.9^{a}
T. pseud	16.8 ± 0.9^{a}	293.0 ± 30.5^{a}	10.9 ± 0.7^{a}	32.2± 8.2^{a}	2.4 ± 0.2^{b}	298.98 ± 23.4^{a}	813.6 ± 87.2^{a}
T. caesp	19.9 ± 2.4^{a}	236.0 ± 26.6^{bc}	13.8 ± 0.6^{a}	39.3 ± 2.7^{a}	6.1 ± 0.2^{c}	229.70 ± 21.5^{b}	590.5 ± 166.3^{a}
AA			6.7 ± 0.7^{b}	-		228.0 ± 20.7^{b}	7.8 ± 1.0^{b}
BHA			-	16.0 ± 2.0^{b}	0.4 ± 0.02^{d}		

Valores medios ± SD. El análisis estadístico se realizó mediante ANOVA de una vía, seguido de la prueba de Tukey. En cada fila, letras diferentes significan diferencias estadísticamente significativas ($p < 0.05$). CFT: compuestos fenólicos totales; EAG: equivalentes de ácido gálico; LOX-5: enzima 5-lipoxigenasa; PR: poder reductor; *T. h-b*: *T. herba-barona; T. pseud*: *T. pseudolanuginosus; T. caesp*: *T. caespititius*; AA: ácido ascórbico; BHA: hidroxianisol butilado.

Debe tenerse en cuenta que estos tres extractos acuosos tenían un contenido fenólico total considerablemente más alto que los encontrados por otros autores en distintas plantas de tomillo. En particular, los extractos de *T. serpyllum* a 50ºC y 100ºC contenían 79.02 y 91.07 mg EAG/g extracto, respectivamente [16], mientras que los extractos

acuosos obtenidos de las hojas de *Thymus zygis* a 20ºC demostraran 25.8 mg de EAG/g de muestra seca [9].

Los constituyentes fenólicos individuales de los tres extractos acuosos de tomillo fueron determinados mediante UHPLC-DAD-ESI-MS^n, teniendo en cuenta los datos recogidos de los espectros UV-Vis y MS de los picos cromatográficos eluidos (Tabla 2) y comparación con los compuestos estándares y/o comparación con los datos de la literatura.

Tabla 2. Identificación y cuantificación a través de UHPLC de las fracciones obtenidas por UHPLC-DAD-MS^n de extractos de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius*

Fracción	**TR** (min)	**λmax** (nm)	**Compuesto**	**[M-H]-**	**(mg/g extracto)**		
					T. h-b	***T. pseud***	***T. caesp***
1	1.3	270	Acido quinico[A]	191	D	D	D
2	1.6	278	Ácido siringico-O-hexósido[B]	359	D	D	D
3	1.8	281	Danshensu[B]	197	D	D	D
		292, 323	4-*O*-ácido cafeoilquinico[B]	353	-	D	D
4	2.2	286, 322	*t*-5-*O*- ácido cafeoilquinico[A]	353	D	6.4 ± 0.4	D
5	2.4	271, 333	Apigenina di-*C*-glucósido[B]	593	D	D	4.0 ± 0.2
6	3.3	289, 321	Ácido cafeico[A]	179	4.3 ± 0.1	D	D
7	3.4	287, 318	Derivado del ácido salvianólico F[B]	375	D	D	D
		277	Derivado del ácido rosmarínico[B]	377	-	-	D
8	3.7	283	Eriodictiol-*O*-glucósido[A]	449	1.9 ± 0.01	-	-
9	4.1	281, 342	Quercetina-*O*-glucurónido[B]	477	2.3 ± 0.1	3.4 ± 0.04	1.1 ± 0.08
10	4.6	341	Luteolina-C-glucósido[A]	447	5.1 ± 0.1	2.9 ± 0.02	-
11	5.1	282	Sulfato de ácido rosmarínico[B]	439	-	-	D
12	5.6	253, 287, 312	Ácido salvianólico I[B]	537	-	D	D
13	5.7	289, 318	Derivado del ácido salvianólico F[B]	519	D	-	-
14	6.2	254, 266, 345	Luteolina-*O*-rutinósido[B]	593	-	-	2.2 ± 0.1
15	6.8	281, 331	Luteolina-*O*-glucurónido (isómero 1)[B]	461	4.4 ± 0.02	54.1 ± 0.6	17.3 ± 1.1
16	7.0	255, 265, 345	Luteolina-*O*-glucurónido (isómero 2)[B]	461	10.5 ± 0.2	7.1 ± 0.2	6.8 ± 0.4
17	7.3	261, 331	Apigenina-*O*-glucósido (isómero 1)[A]	431	-	0.9 ± 0.15	-
18	8.0	285, 333	Derivado del ácido salvianólico C[B]	553	-	D	-
19	8.3	254, 283, 344	Ácido salvianólico B (isómero 1)[B]	717	-	-	6.9 ± 0.5
		289, 318	Diidro- Ácido salvianólico B (isómero 1)[B]	715	10.8 ± 0.1	-	-

20	8.5	289, 337	Crisoeriol-*O*- glucósido[B]	461	D	D	-
21	9.0	228, 282, 331	Apigenina-*O*-glucósido (isómero 2)[B]	431	D	-	-
22	9.0	252, 267, 342	Crisoeriol-*O*-rutinósido[B]	607	-	-	D
23	9.1	267, 333	Apigenina-*O*- glucurónido[B]	445	2.10 ± 0.03	8.30 ± 0.05	1.97 ± 0.1
24	9.6	287, 325	Ácido rosmarínico[A]	359	55.8 ± 2.8	40.2 ± 0.9	43.2 ± 3.2
25	9.8	287, 311	3′-*O*-(8″-Z-Cafeoil) Ácido rosmarínico (isómero 1)[B]	537	D	D	D
26	9.0	289, 319	Diidro - Ácido salvianólico B (isómero 2)[B]	715	D	-	-
27	10.1	288, 326	Ácido salvianólico B (isómero 2)[B]	717	D	D	D
		287, 324	Ácido salvianólico K[B]	555	D	10.5 ± 0.1	-
28	10.7	290, 323	3′-*O*-(8″-Z-Cafeoil) Ácido rosmarínico (isómero 2)[B]	537	12.0 ± 0.2	D	D
29	12.2	288, 322	Cafeoil ácido rosmarínico (isómero 1)[B]	537	10.5 ± 0.06	D	D
30	12.5	287, 328	Cafeoil ácido rosmarínico (isómero 2)[B]	537	4.2 ± 0.1	-	-
31	12.8	288, 323	Cafeoil ácido rosmarínico (isómero 3)[B]	537	-	D	-
32	13.3	287, 323	3′-*O*-(8″-Z-Cafeoil) ácido rosmarínico (isómero 3)[B]	537	D	D	D
			Classes de Compuestos Fenolicos		97.6 ± 2.6[a]	57.1 ± 1.3[b]	50.0 ± 3.8[c]
					22.0 ± 0.3[a]	73.3 ± 1.0[b]	32.2 ± 2.0[c]
					2.3 ± 0.1[a]	3.60 ± 0.04[b]	1.1 ± 0.1[c]
					1.9 ± 0.01	-	-
					123.9 ± 2.8[a]	134.0 ± 2.4[b]	83.4 ± 5.8[c]

T. h-b: *T. herba-barona; T. pseud*: *T. pseudolanuginosus; T. caes: T. caespititius;* D: Detectado; TR: tiempo de retención; [A]La identificación del compuesto se basó en la comparación con el estándar; [B]La identificación del compuesto se basó en la interpretación de espectros UV y datos de EM, más la comparación con la literatura; Valores medios ± SD de tres ensayos independientes; El análisis estadístico fue realizado por one-way ANOVA (teste de Tukey). En cada fila diferentes letras significan diferencias estadísticamente significativas ($p < 0.05$).

Es conocido que las plantas de *Thymus* presentan grandes cantidades de ácido rosmarínico [1]. En este estudio, el ácido rosmarínico fue un componente fenólico importante en los tres extractos de tomillo, representando 55.8, 40.2 y 43.2 mg/g en *T. herba-barona*, *T. pseudolanuginosus* y *T. caespititius*, respectivamente. El ácido rosmarínico fue claramente menos representativo en el extracto de *T. pseudolanuginosus* (30% del total de fenólicos cuantificados) en comparación con los otros dos (45-52% del total de fenólicos cuantificados). Esta diferencia se debió principalmente a la alta abundancia de luteolina-*O*-glucurónido (6.8 min, $[M-H]^-$ at m/z

461→285) en el extracto de *T. pseudolanuginosus* (54.1 mg/g), mientras que sus niveles fueron de 17.3 y 4.4 mg/g en los extractos de *T. caespititius* y *T. herba-barona*, respectivamente. En general, se observó una elevada expresión de los dos derivados de la luteolina-*O*-glucurónido en la especie *T. pseudolanuginosus* (61.2 mg/g de extracto) que representaron 46% de sus compuestos fenólicos totales. Esto indica la riqueza en flavonas del *T. pseudolanuginosus* y una clara diferenciación de esto extracto en relación a *T. herba-barona* y *T. caespititius.* Es importante anotar que los derivados de la luteolina-*O*-glucurónido y otros glucósidos de flavona también se han descrito previamente para otras especies de *Thymus*, pero aun así, sus niveles fueron más altos en *T. pseudolanuginosus* que los publicados anteriormente (8-14 mg/g de planta) [1,19,23].

Además del ácido rosmarínico, los restantes derivados de lo ácido cafeico estaban presentes en las tres especies de *Thymus*, siendo particularmente abundante en extractos de *T. herba-barona* (Tabla 2). Por otro lado, los ácidos salvianólicos K y B se encontraron en los extractos de *T. pseudolanuginosus* y *T. caespititius*, con niveles de 10.5 y 6.9 mg/g de extracto, respectivamente.

3.2. Actividad antioxidante

La sobreproducción de oxidantes es responsable de la patogénesis de muchas enfermedades crónicas. A su vez, los distintos fitoquímicos de los alimentos y las plantas medicinales exhiben propiedades antioxidantes que podrían ser beneficiosas para contrarrestar el estrés oxidativo [10]. En este contexto, los extractos acuosos de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius* se investigaron por su capacidad antioxidante, por distintos métodos *in vitro*, a saber, DPPH$^{\bullet}$, poder reductor y ensayos de blanqueo del β-caroteno (Tabla 1). El método de captación de DPPH$^{\bullet}$ evalúa la capacidad de los extractos para atrapar los radicales libres sintéticos DPPH$^{\bullet}$, mientras que el método de la potencia reductora mide la conversión de Fe^{3+} a Fe^{2+}, y el método de blanqueo β-caroteno mide la capacidad de inhibir la peroxidación lipídica.

Teniendo en cuenta los resultados obtenidos, se puede concluir que los tres extractos de *Thymus* mostraron una alta capacidad antioxidante, lo cual fue particularmente evidente en las pruebas de DPPH$^{\bullet}$ y del poder reductor, para las cuales los valores de EC_{50} son 1.6-2.0 más altos que los de los compuestos comerciales de referencia. Entre los tres extractos, había una tendencia a una mejor actividad de *T. pseudolanuginosus* (EC_{50} de 10.9 y 32.2 μg/mL en los métodos DPPH$^{\bullet}$ y poder reductor, respectivamente), aunque las diferencias no fueron estadísticamente significativas.

La alta capacidad antioxidante aquí descrita está de acuerdo con los datos de la literatura encontrados para los extractos acuosos de *T. serpyllum* L. con bajos valores de EC_{50} en DPPH$^{\bullet}$ (13.8 y 11.8 µg/mL obtenidos en 50ºC y 100ºC, respectivamente) [16] y para los extractos etanólicos de otras especies de tomillo, que presentaron valores EC_{50} en el ensayo del DPPH$^{\bullet}$, 1.8-3.6 más alto que los de los compuestos comerciales de referencia [15]. Resultados menos prometedores se habían descrito previamente para otras especies de tomillo usando el método DPPH$^{\bullet}$, a saber, en extractos polares de *Thymus capitatus* [24] y de *Thymus pubescens*, *Thymus kotschyanus* and *Thymus daenensis* [25], cuyos valores de EC_{50} fueron 6-9 veces superiores a los de los controles positivos utilizados. La capacidad de especies de *Thymus* para reducir Fe^{3+} a Fe^{2+} no está bien documentada, sin embargo Kindl *et al.* [15] habían descrito una fuerte capacidad en extractos etanólicos de *Thymus longicaulis, Thymus praecox, Thymus pulegioides, T. serpyllum, Thymus striatus* y *T. vulgaris* (EC_{50}=11.4-15.1 µg/mL), con los mejores resultados registrados para *T. pulegioides*, cuyo EC_{50} fue solo 1.7 más alto que el control de referencia [15].

La oxidación de β-caroteno fue inhibida efectivamente por *T. pseudolanuginosus* (EC_{50}=2.4 µg/mL), seguido de *T. caespititius* (EC_{50}=6.1 µg/mL), mientras que *T. herba-barona* se mostró ineficaz (Table 1). Los datos de la literatura también señalan una actividad prometedora en el test de oxidación de β-caroteno para otros tomillos. En particular, Iauk *et al.* [24] describieron valores de EC_{50} para un extracto metanólico de *T. capitatus* que fue cercano a 0.7 µg/mL en 30 min y 1.9 µg/mL en 60 min. Se observaron valores más altos para los extractos etanólicos de *T. daenensis*, *T. kotschyanus* y *T. pubescens* (EC_{50}= 23.7, 35.2 y 92.9 µg/mL, respectivamente) [25].

La información combinada de los tres métodos antioxidantes junto con la de los niveles de compuestos fenólicos sugiere que estos últimos constituyentes no son los únicos actores que dictan el potencial antioxidante de los extractos. La alta abundancia de compuestos fenólicos, y particularmente de las flavonas, en el extracto de *T. pseudolanuginosus*, se puede relacionar con su capacidad antioxidante superior en comparación con la de los extractos restantes. Por otra parte, el contenido fenólico directo y la actividad antioxidante no se observaron para las dos especies de plantas restantes en el ensayo de blanqueo de β-caroteno. Este fenómeno puede deberse a la presencia de componentes inductores oxidativos en el extracto de *T. herba-barona*, además de los compuestos fenólicos antioxidantes, que mitigan la actividad de este último.

3.3 Actividad antiinflamatoria

Debido a la relevancia de los procesos inflamatorios en la aparición de numerosas enfermedades (cáncer, Alzheimer, insuficiencia cardíaca, accidente cerebrovascular isquémico y otros), la búsqueda de extractos naturales de baja toxicidad capaces de contrarrestar a los principales elementos mediadores de la inflamación se ha incrementado considerablemente en los últimos años [31,32]. Los extractos acuosos de las tres especies de plantas se examinaron para determinar su capacidad para contrarrestar el incremento de $NO^{\bullet}$ (es decir, un mediador proinflamatorio principal producido por fagocitos) y la actividad de la 5-LOX, la enzima que controla la producción de los leucotrienos proinflamatorios [6,26]. Como se detalla en la Tabla 1, todos los extractos mostraron una alta capacidad de reducir los niveles de $NO^{\bullet}$. Este efecto fue más evidente para *T. caespititius*, que exhibió la misma potencia que el ácido ascórbico (EC_{50}=229.7 µg/mL y 228.0 µg/mL, respectivamente). *T. caespetitius* también fue el más relevante entre las tres muestras con respecto a la capacidad para inhibir la actividad 5-LOX (EC_{50}=590.5 µg/mL). Por lo tanto, los resultados sugieren que los extractos acuosos de tomillo de las plantas seleccionadas podrían tener aplicaciones potenciales como agentes antiinflamatorios, actuando a través de capacidades antiradicales hacia $NO^{\bullet}$. El hecho de que esta capacidad no esté directamente asociada al contenido fenólico de los extractos (el extracto de *T. caespititius* fue el menos rico en compuestos fenólicos) también sugiere que los compuestos no fenólicos pueden tener funciones importantes en el efecto antiinflamatorio.

Por lo que sabemos, la capacidad de la eliminación de $NO^{\bullet}$ de extractos polares de *Thymus* solo se ha informado previamente para extractos etanólicos de seis especies de *Thymus*: *T. serpyllum* subsp. *serpyllum* (EC_{50}=176.6 µg/mL), *T. praecox* subsp. *polytrichus* (EC_{50} = 139.0 µg/mL), *T. vulgaris* (EC_{50} = 97.9 µg/mL), *T. striatus* (EC_{50} = 91.1 µg/mL), *T. longicaulis* (EC_{50} = 71.6 µg/mL), *T. pulegioides* (EC_{50} = 69.8 µg/mL) [15], que mostraron valores EC_{50} 1.3-3.2 veces más altos que el compuesto estándar (Trolox), mientras que la actividad de inhibición de 5-LOX no fue explorada.

3.4. Actividad antibacteriana

La resistencia a los antibióticos se viene asociando a enfermedades y altas tasas de mortalidad [27]. Las investigaciones sobre nuevas sustancias antimicrobianas, como los compuestos naturales purificados de plantas, pueden servir de estrategia terapéutica para la obtención de fármacos alternativos y de nueva generación con baja toxicidad. En este campo, los compuestos fenólicos han ido ganando importancia [28]. La actividad antibacteriana de la familia *Lamiaceae* ha sido ampliamente estudiada, con resultados

prometedores para los aceites esenciales de *Thymus* [29,30] mientras que se ha avanzado menos en el conocimiento de extractos polares de estas plantas.

Los datos recopilados mostraron que, entre las 5 cepas analizadas (Tabla 3), *S. aureus* fue la más sensible a los tres extractos de tomillo, que fueron capaces de inhibir tanto su crecimiento como su viabilidad a 0.6, 1.6 y 3.5 mg/ml para *T. herba-barona, T. pseudolanuginosus* y *T. caespititius,* respectivamente. Estos resultados son consistentes con los descritos por Benbelaïd et al. [21] para un extracto acuoso de *T. lanceolatus*, que exhibió un valor de CIM de 1.0 mg/ml para *S. aureus*, evaluado mediante el método de microdilución en caldo. Además, los resultados obtenidos utilizando el método de dilución en agar, mostraron valores de CIM de 0,78 mg/ml para extractos etanólicos de *T. caramanicus*, cuando se expusieron a *S. aureus* [20].

Table 3. CIM (mg/ml) and CBM (mg/ml) de extractos de planta y nisina (mg/ml) contra cinco patógenos

Bacteria	*T. herba-barona*		*T. pseudolanuginosus*		*T. caespititius*		Nisina	
	CBM	CIM	CBM	CIM	CBM	CIM	CBM	CIM
S. typhimurium	5.0	5.0	>6.5	6.5	>7.0	7.0	0.5	0.5
S. epidermidis	5.0	5.0	6.5	6.5	7.0	3.5	<0.03	<0.03
S. aureus	0.6	0.6	1.6	1.6	3.5	3.5	0.25	0.25
E. coli	>5.0	5.0	>6.5	6.5	7.0	7.0	0.5	0.5
P. aeruginosa	>5.0	5.0	>6.5	6.5	>7.0	7.0	1.0	0.5

Valores medios; CIM: concentración inhibitoria mínima; CBM: concentración bactericida mínima

Las tres especies de *Thymus* estudiadas, también mostraron un efecto antibacteriano relevante contra las bacterias restantes analizadas, aunque con menos potencia que el conservante de alimentos nisina. Más concretamente, se registraron valores de CIM de 5.0 y 6.5 mg/ml para *T. herba-barona* y *T. pseudolanuginosus*, respectivamente, contra *S. typhimurium, S. epidermidis, E. coli* y *P. aeruginosa*. A pesar de que *T. caespititius* fue, en general, el extracto menos activo, su actividad hacia *S. epidermidis* (CIM=3.5 mg/ml) fue más efectiva que las restantes. Las concentraciones de CIM que se recogen en este estudio son cercanas a las publicadas previamente por otros autores para los extractos polares de *T. vulgaris*, *Thymus caramanicus* [20] y *T. lanceolatus* [21] contra una panel semejante de bacterias.

En general, los resultados mostraron que los extractos de tomillo no eran letales para algunas bacterias Gram- (*S. typhimurium , E. coli* y *P. aeruginosa*), con una CBM alta (Tabla 3). Además, las bacterias Gram- fueron más resistentes (CIM entre 5.0 y 7.0 mg/ml) que las bacterias Gram +, particularmente *S. aureus* (CIM en el rango de 0.6 -

3.5 mg/ml). Estos resultados están de acuerdo con los de otros autores, que investigaron extractos acuosos de *T. lanceolatus* (CIM=4.0 mg/ml contra *S. typhimurium* y CIM=0.83 mg/ml contra *S. aureus*) [21]. La misma evidencia se observó en varias investigaciones que analizaron el efecto de aceites esenciales extraídos de plantas *Thymus* [29,30].

4. Conclusiones

Este trabajo describe en detalle la composición fenólica de decocciones de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius*, así como sus actividades antioxidante, antiinflamatoria y antimicrobiana. Los tres extractos acuosos se caracterizaron principalmente por la presencia de ácidos fenólicos y flavonoides, que en parte parecen estar relacionados con sus actividades biológicas. *T. pseudolanuginosus* presentó los mejores resultados antioxidantes en los tres métodos utilizados. Los extractos de tomillos seleccionados mostraron actividad antibacteriana contra el panel de bacterias utilizado en este estudio, especialmente las cepas de *S. aureus*, que en general fueron también las más sensibles. *T. caespititius* tiene potencial antiinflamatorio, basado en su prometedora actividad inhibitoria en la producción de $NO^{\bullet}$. Teniendo en cuenta la creciente demanda de extractos bioactivos de origen botánico, este trabajo abre una puerta para la expansión de la explotación comercial de estas especies de tomillo.

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Article

Health-Promoting Effects of *Thymus herba-barona*, *Thymus pseudolanuginosus*, and *Thymus caespititius* Decoctions

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Received: 4 August 2017; Accepted: 26 August 2017; Published: 31 August 2017

Abstract: *Thymus herba-barona*, *Thymus pseudolanuginosus*, and *Thymus caespititius* decoctions were screened for their phenolic constituents, along with their potential antioxidant, anti-inflammatory, and antibacterial activities. The total phenolic compounds in the extracts of the three plants ranged from 236.0 ± 26.6 mgGAE/g (*T. caespititus*) to 293.0 ± 30.5 mgGAE/g of extract (*T. pseudolanuginosus*), being particularly rich in caffeic acid derivatives, namely rosmarinic acid and its structural isomers, as well as flavones, such as luteolin-*O*-glucuronide. The *T. pseudolanuginosus* extract presented the best DPPH radical scavenging ability (EC_{50} = 10.9 ± 0.7 μg/mL), a high reducing power (EC_{50} = 32.2 ± 8.2 μg/mL), and effectively inhibited the oxidation of β-carotene (EC_{50} = 2.4 ± 0.2 μg/mL). The extracts also showed $NO^{\bullet}$ scavenging activity close to that of ascorbic acid, and thus might be useful as anti-inflammatory agents. In addition, they exhibited antibacterial activity against gram-negative and gram-positive bacteria. *Staphylococcus aureus* strains were the most sensitive bacteria to thyme extracts, with minimum inhibitory concentration and minimum bactericidal concentration values in the range of 0.6–3.5 mg/mL. Overall, this work is an important contribution for the phytochemical characterization and the potential antioxidant, anti-inflammatory, and antimicrobial activities of these three *Thymus* species, which have been poorly explored.

Keywords: thymus; thyme; LC-MS; mass spectrometry; phenolic; antioxidant; anti-inflammatory; antiradicalar; antimicrobial activity

1. Introduction

The *Thymus* genus encloses about 350 species, which are particularly well adapted to the hot and dry climate of the Mediterranean region and widespread in the arid parts of the Iberian Peninsula [1,2]. As for the vast majority of Lamiaceae plants, *Thymus* are recognized as being strongly aromatic and are widely used as spices to enhance sensory attributes such as the taste and aroma of foods [1]. These properties highlight the potential application of *Thymus* plants in the food industry, e.g., in meat, butter, chewing gum, liqueurs, ice cream, and candy production. Besides flavouring, their usage also improves the preservation of food, contributing to the prevention of food oxidation and color changes [3,4]. Likewise, these plants have some potential applications in pharmaceutical and cosmetic industries due to their biological and medicinal benefits. They are largely used in the manufacturing

of perfumes, pharmaceutical products, and toilet articles [4–7]. Although these applications have been mostly associated with essential oils, nowadays, *Thymus* polar extracts are an attractive target for the screening of health-promoting properties for possible industrial applications in food, cosmetics, or pharmaceutical industries, among others [1,8].

Several authors have demonstrated that Lamiaceae, and particularly *Thymus* plants, are rich in bioactive phytochemicals, including phenolic acids and flavonoids [1,3,9,10], which in turn, have been shown to reduce the risk of diseases due to their extensive biological properties, including those which are antioxidant, cardioprotective, anticancer, anti-ageing, anti-inflammatory, and antibacterial [5,6,8,11].

The screening of the potential bioactive effects of *Thymus* polar extracts has been mainly conducted for *Thymus vulgaris* [12–15] and *Thymus serpyllum* (wild thyme) [2,16], although other species, including *Thymus pulegioides* [4,15], *Thymus praecox* [15], *Thymus sipyleus* [17], *Thymus striatus* [15], *Thymus longicaulis* [15], *Thymus mastichina* [18], have also been studied with respect to their antioxidant activities. Overall, aqueous or alcoholic extracts from *Thymus* have been shown to exhibit strong antioxidant activities in in vitro [17,19] or in vivo models [20]. Moreover, other effects reported in the polar extracts of thyme species include antimicrobial [21,22], antidiabetic [8,19], neuroprotective [15], and inflammatory properties [23].

In contrast to the above mentioned studies, the potential applications of the polar extracts from less-widespread *Thymus* like *Thymus herba-barona*, *Thymus pseudolanuginosus*, and *Thymus caespititius* remain unexplored. These three species are particularly known for being well-adapted to the Atlantic climate, thus growing spontaneously in the Iberian Peninsula and Mediterranean islands. As reported, their essential oils exhibit antioxidant, antimicrobial, and insecticidal effects and can potentially be used as preservatives in storage products for food flavouring, and as deodorants and disinfectants [24,25]. The present study aims to contribute to the clarification of phenolic constituents of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius*, as well as to exploit their free radical scavenging, antibacterial, and anti-inflammatory activities.

2. Results and Discussion

2.1. Phenolic Compounds in Thymus Aqueous Extracts

The mass yield of thyme aqueous extracts ranged from 15% to 20%, with *T. caespititius* being the most predominant (Table 1). In turn, the latter showed a lower total phenolic content (236 $\pm$ 26.6 mg GAE/g extract, equivalent to 47.0 mg GAE/g dry plant) in comparison to the mean values obtained for the *T. herba-barona* and *T. pseudolanuginosus* extracts (273 and 293 mg GAE/g extract or 41.6 and 49.2 mg GAE/g dry plant, respectively). However, it is important to note that these three aqueous extracts had a considerably higher total phenolic content than those found by other authors in distinct *Thymus* plants. In particular, aqueous extracts obtained from wild thyme (*T. serpyllum*) at 50 and 100ºC were found to contain 79.02 $\pm$ 6.62 and 91.07 $\pm$ 9.25 mg GAE/g extract, respectively [16], while aqueous extracts obtained from *Thymus zygis* leaves at 20ºC produced a 25.8 $\pm$ 2.0 mg GAE/g dry sample [3]. In addition, the phenolic content in phosphate buffer or hydromethanolic extracts from *T. vulgaris* were reported to account for 2.1 $\pm$ 0.1 mg GAE/g fresh leaves [26] or 19.2 $\pm$ 0.3 mg GAE/g of fresh weight [14], respectively.

Individual phenolic constituents of the three thyme aqueous extracts were elucidated by ultra high performance liquid chromatography coupled to diode array detector and an a electrospray mass spectrometer (UHPLC-DAD-ESI-MS^n), taking into consideration the gathered ultraviolet-visible (UV-Vis) and mass spectrometry (MS) spectra data of the eluted chromatographic peaks (Figure 1, Table 2) and in comparison to those of standard compounds and/or in comparison to literature data.

Table 1. Yield of extraction (%), total phenolic content (mg GAE/g of extract), and antioxidant and anti-inflammatory activities (EC_{50}, µg/mL) of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* aqueous extracts.

Plant Extract	Yield (%)	TPC	DPPH•	RP	β-Carotene	NO•	5-LOX
T. herba-barona	15.3 ± 1.80 ^{a}	273 ± 16.6 ^{a,c}	11.6 ± 0.90 ^{a}	35.1± 4.50 ^{a}	>26.70 ^{a}	286 ± 32.6 ^{a}	841 ± 138 ^{a}
T. pseudolanuginosus	16.8 ± 0.90 ^{a}	293 ± 30.5 ^{a}	10.9 ± 0.70 ^{a}	32.2± 8.20 ^{a}	2.40 ± 0.20 ^{b}	299 ± 23.4 ^{a}	814 ± 87.2 ^{a}
T. caespititius	19.9 ± 2.40 ^{a}	236 ± 26.6 ^{b,c}	13.8 ± 0.60 ^{a}	39.3 ± 2.70 ^{a}	6.10 ± 0.20 ^{c}	230 ± 21.5 ^{b}	591 ± 166 ^{a}
AA			6.70 ± 0.70 ^{b}	–		228 ± 20.7 ^{b}	7.80 ± 1.00 ^{b}
BHA			–	16.0 ± 2.00 ^{b}	0.40 ± 0.02 ^{d}		

Mean values ± standard deviations. Statistical analysis was performed by one-way ANOVA, followed by a Tukey test. In each column, different letters (a–d) stand for significant statistical different data ($p < 0.05$). TPC: Total Phenolic Compounds; RP: Reducing Power; AA: Ascorbic acid; BHA: Butylated hydroxyanisole; LOX: lipoxygenase.

Note that despite the fact that *Thymus* plants are generally known for their richness in rosmarinic acid [1], the specific phenolic composition of thyme extracts is dependent on several factors, including the botanical species and the applied extraction conditions. To the best of our knowledge, there is no reported literature regarding the phenolic composition of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* extracts.

Rosmarinic acid was a major phenolic component in the three thyme extracts, accounting for 55.8 ± 2.8 mg/g in *T. herba-barona* and 40.2 ± 0.9 and 43.2 ± 3.2 mg/g in *T. pseudolanuginosus* and *T. caespititius*, respectively. Despite the common abundance, rosmarinic acid was clearly less representative in the *T. pseudolanuginosus* extract (30% of total quantified phenolics) in comparison to the other two, in which it amounted for 45–52% of the total quantified phenolics. This difference was mainly due to the high abundance of luteolin-*O*-glucuronide (6.8 min, $[M-H]^-$ at *m/z* 461→285) in the *T. pseudolanuginosus* extract, which accounted for 54.1 ± 0.6 mg/g, while its levels were only 17.3 ± 1.1 mg/g and 4.4 ± 0.02 mg/g in the *T. caespititius* and *T. herba-barona* extracts, respectively. Globally, the two *O*-glucuronide derivatives of luteolin eluted in fractions 15 and 16 (RT 6.8 and 7.0 min respectively) in the *T. pseudolanuginosus* extract accounted for 61.2 mg/g of the extract and represented 46% of its total phenolics. This caused a clear differentiation between the extracts, with *T. pseudolanuginosus* phenolic components being mainly represented by flavones. Note that *O*-glucuronide derivatives of luteolin and other flavone glycosides such as luteolin-*C*-glucoside, apigenin-*O*-glucuronide and apigenin-*O*-glucoside herein detected have also been previously described for other *Thymus* species, but still, their levels, particularly those of luteolin-*O*-glucuronide derivatives, are higher in *T. pseudolanuginosus* than in previously reported data (8–14 mg/g of dry plant) [1,17,27].

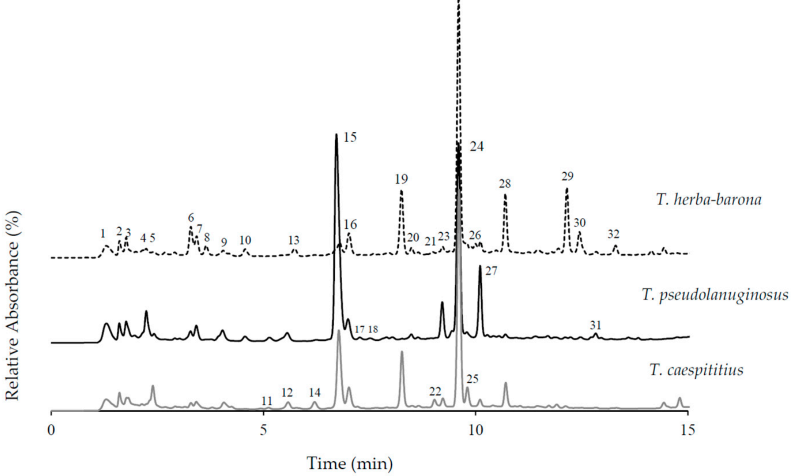

Figure 1. Chromatographic profiles at 280 nm of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* aqueous extracts (The numbers in the figure correspond to the LC-MS^n fractions indicated in Table 2).

Table 2. Identification and quantification of UHPLC (ultra high performance chromatography) eluting fractions by UHPLC-DAD-MS^n of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* aqueous extracts.

Fraction	RT (min)	λmax (nm)	Compound	[M-H]$^-$	Main Fragments ESI-MS^n	(mg/g Extract)		
						T. h-b	*T. pseud*	*T. caesp*
1	1.3	270	Quinic acid [A]	191	MS2 [191]: 111, 173	D	D	D
2	1.6	278	Syringic acid-*O*-hex [B]	359	MS2 [359]: 197, 179, 161, 153, 135	D	D	D
3	1.8	281 292, 323	Danshensu [B] 4-*O*-CQA [B]	197 353	MS2 [197]: 179 MS2 [353]: 173, 179, 191	D –	D D	D D
4	2.2	286, 322	*t*-5-*O*-CQA [A]	353	MS2 [353]: 191 , 179, 161, 135, 119	D	6.4 ± 0.4	D
5	2.4	271, 333	Apigenin di-*C*-glc [B]	593	MS2 [539]: 473, 353, 383, 503, 575, 297	D	D	4.0 ± 0.2
6	3.3	289, 321	Caffeic acid [A]	179	MS2 [179]: 135, 151, 161, 107, 97	4.3 ± 0.1	D	D
7	3.4	287, 318 277	SA F der [B] RA der [B]	375 377	MS2 [375]: 313, 269, 179, 135, MS3 [313]: 269, 161 MS2 [377]: 359; MS3 [359]: 161, 179, 197, 223, 133	D –	D –	D D
8	3.7	283	Eriodictyol-*O*-glc [A]	449	MS2 [449]: 287, 269, 259, 267	1.9 ± 0.01	–	–
9	4.1	281, 342	Quercetin-*O*-glcA [B]	477	MS2 [477]: 301, 343, 397	2.3 ± 0.1	3.4 ± 0.04	1.1 ± 0.08
10	4.6	341	Luteolin-*C*-glc [A]	447	MS2 [447]: 357, 285, 327	5.1 ± 0.1	2.9 ± 0.02	–
11	5.1	282	RA sulfate [B]	439	MS2 [439]: 259, 421, 225, 371, 359, 197; MS3 [259]: 161	–	–	D
12	5.6	253, 287, 312	SA I [B]	537	MS2 [537]: 339, 493; MS3 [339]: 295, 229, 293	–	D	D
13	5.7	289, 318	SA F der [B]	519	MS2 [519]: 475, 313; MS3 [475]: 313, 269, 179, 431	D	–	–
14	6.2	254, 266, 345	Luteolin-*O*-rut [B]	593	MS2 [593]: 285	–	–	2.2 ± 0.1
15	6.8	281, 331	Luteolin-*O*-glcA (isom 1) [B]	461	MS2 [461]: 285, 175; MS3 [285]: 267, 239, 241, 213, 185	4.4 ± 0.02	54.1 ± 0.6	17.3 ± 1.1
16	7.0	255, 265, 345	Luteolin-*O*-glcA (isom 2) [B]	461	MS2 [461]: 285; MS3 [285]: 241, 199, 175, 151, 267	10.5 ± 0.2	7.1 ± 0.2	6.8 ± 0.4
17	7.3	261, 331	Apigenin-*O*-glc (isom 1) [A]	431	MS2 [431]: 269	–	0.9 ± 0.15	–
18	8.0	285, 333	SA C der [B]	553	MS2 [553]: 491, 399, 179, 429, 473; MS3 [491]: 473	–	D	–

Table 2. *Cont.*

Fraction	RT (min)	λmax (nm)	Compound	[M − H]⁻	Main Fragments ESI–MSⁿ	(mg/g Extract)		
						T. h-b	***T. pseud***	***T. caesp***
19	8.3	254, 283, 344	SA B (isom1) [B]	717	MS2 [717]: 519, 475, 339; MS3 [519]: 475, 339	–	–	6.9 ± 0.5
		289, 318	Dedihydro-SA B (isom 1) [B]	715	MS2 [715]: 313, 627, 671, 269; MS2 [313]: 179, 135	10.8 ± 0.1	–	–
20	8.5	289, 337	Chrysoeriol-*O*-glc [B]	461	MS2 [461]: 299, 284; MS3 [299]: 284	D	D	–
21	9.0	228, 282, 331	Apigenin-*O*-glc (isom 2) [B]	431	MS2 [431]: 269; MS3 [269]: 225, 149, 117, 183, 167, 199	D	–	–
22	9.0	252, 267, 342	Chrysoeriol-*O*-rut [B]	607	MS2 [607]: 299, 284 ; MS3 [299]: 284	–	–	D
23	9.1	267, 333	Apigenin-*O*-glcA [B]	445	MS2 [445]: 269, 175	2.1 ± 0.03	8.3 ± 0.05	1.97 ± 0.1
24	9.6	287, 325	RA [A]	359	MS2 [359]: 161, 179, 197, 223	55.8 ± 2.8	40.2 ± 0.9	43.2 ± 3.2
25	9.8	287, 311	3′-*O*-(8′′-Z-Caffeoyl) RA (isom 1) [B]	537	MS2 [537]: 493, 515, 375, 357, 339, 313, 197	D	D	D
26	9.0	289, 319	Dedihydro- SA B (isom 2) [B]	715	MS2 [715]: 313, 671, 627, 269	D	–	–
27	10.1	288, 326	SA B (isom 2) [B]	717	MS2 [717]: 519, 357, 555 MS3 [519]: 357, 475, 295	D	D	D
		287, 324	SA K [B]	555	MS2 [555]: 493, 357, 393, 313; MS3 [493]: 359, 313, 161	D	10.5 ± 0.1	–
28	10.7	290, 323	3′-*O*-(8′′-Z-Caffeoyl) RA (isom 2) [B]	537	MS2 [537]: 493, 359; MS3 [493]: 359, 313, 295, 161	12.0 ± 0.2	D	D
29	12.2	288, 322	Caffeoyl RA (isom 1) [B]	537	MS2 [537]: 375, 493, 359, 519	10.5 ± 0.06	D	D
30	12.5	287, 328	Caffeoyl RA (isom 2) [B]	537	MS2 [537]: 439, 519, 357, 493, 323, 197	4.2 ± 0.1	–	–
31	12.8	288, 323	Caffeoyl RA (isom 3) [B]	537	MS2 [537]: 519, 359, 357, 339, 235, 493; MS3 [519]: 357	–	D	–
32	13.3	287, 323	3′-*O*-(8′′-Z-Caffeoyl) RA (isom 3) [B]	537	MS2 [537]: 493, 375, 359; MS3 [493]: 359, 197	D	D	D
			Phenolic compounds groups		*Caffeic acid and derivatives*	97.6 ± 2.6 [a]	57.1 ± 1.3 [b]	50.0 ± 3.8 [c]
					Flavones	22.0 ± 0.3 [a]	73.3 ± 1.0 [b]	32.2 ± 2.0 [c]
					Flavonols	2.3 ± 0.1 [a]	3.6 ± 0.04 [b]	1.1 ± 0.1 [c]
					Flavanones	1.9 ± 0.01	–	–
					Total	123.9 ± 2.8 [a]	134.0 ± 2.4 [b]	83.4 ± 5.8 [c]

T. h-b: *Thymus herba-barona*; *T. pseud*: *Thymus pseudolanuginosus*; *T. caes: Thymus caespititius*; D: detected; RT: retention time; CQA: caffeoylquinic acid; Der: derivative; Glc: glucoside; GlucA: glucuronide; Hex: hexoside; isom: isomer; RA: rosmarinic acid; Rut: rutinoside; SA: salvianolic acid; [A]compound identification was based on comparison to standard; [B]compound identification was based on interpretation of UV spectral and MS data, plus comparison to literature; Mean values ± standard deviations of three independent assays; Statistical analysis was performed by one-way ANOVA (Tukey's test). In each row, different letters (a–c) stand for significant statistical different data ($p < 0.05$).

Apart from rosmarinic acid, the remaining caffeic acid derivatives represented 8%, 13%, and 34% of the total quantified phenolic compounds in *T. caespititius*, *T. pseudolanuginosus*, and *T. herba-barona* extracts, respectively. Among the three plant species, the latter was clearly the richest in this group of compounds, comprising simple compounds, namely caffeic acid (4.3 ± 0.1 mg/g extract) and *t*-5-*O*-CQA (in vestigial concentrations), as well as several depsides (Table 2), namely 3′-*O*-(8″-*Z*-caffeoyl)rosmarinic acid (a compound previously described in other thyme species [28]) and/or its isomers (MW 538 Da, fractions 25, 28 and 32), together with dihydro-salvianolic acid B (fraction 19; MW 716 Da). On the other hand, salvianolic acids K (fraction 27, $[M - H]^-$ at *m/z* 555 → 493 → 359) and B (fraction 19, $[M - H]^-$ at *m/z* 717 → 519 → 475) were found in the *T. pseudolanuginosus* and *T. caespititius* extracts, with levels of 10.5 ± 0.1 and 6.9 ± 0.5 mg/g of the extract, respectively.

2.2. Antioxidant Capacity

The overproduction of oxidants is responsible for the pathogenesis of many chronic diseases. In turn, distinct phytochemicals from foods and medicinal plants exhibit antioxidant properties that might be beneficial to counteract oxidative events [11]. In this context, the three *Thymus* aqueous extracts were investigated for their antioxidant abilities through distinct in vitro methods, namely DPPH$^•$, reducing power and β-carotene bleaching assays. The DPPH$^•$ scavenging method evaluates the free radical scavenging ability of the plant extracts to trap the synthetic free radicals DPPH$^•$, while the reducing power and β-carotene bleaching methods measure the extract's ability to reduce Fe^{3+} to Fe^{2+} or to inhibit lipidic peroxidation, respectively.

The antioxidant potentialities of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* aqueous extracts are detailed in Table 1, in terms of their EC_{50} values. Considering the results, one can conclude that all three *Thymus* extracts showed a high antioxidant capacity, which was particularly evident in the DPPH$^•$ and reducing power tests, for which the EC_{50} values are 1.6–2.0 higher than those of the reference commercial compounds. Among the three extracts, there is a tendency for the better activity of *T. pseudolanuginosus* (EC_{50} of 10.9 ± 0.7 and 32.2 ± 8.2 µg/mL for DPPH$^•$ and reducing power tests, respectively), although differences are not statistically significant.

The high antioxidant ability herein reported is in accordance with the literature data found for *Thymus* plants. Indeed, low DPPH$^•$ EC_{50} values were previously registered for *T. serpyllum* L. aqueous extracts (EC_{50} of 13.75 ± 1.14 and 11.76 ± 0.25 µg/mL for extracts obtained at 50 and 100°C, respectively) [16] and for the ethanolic extracts from *T. longicaulis*, *T. praecox*, *T. pulegioides*, *T. serpyllum*, *T. striatus*, and *T. vulgaris*, which exhibited DPPH$^•$ EC_{50} values in the range of 3.01–6.01 µg/mL, i.e., 1.8–3.6 higher than those of the reference commercial compounds [15]. Less promising results were previously reported for *Thymus capitatus* methanolic and hexane extracts, with EC_{50} = 44.5 ± 1.9 and 38.2 ± 1.2 µg/mL, respectively (eight to nine times less active than the positive control EC_{50} = 5.0 ± 0.8) [29] and for hydroalcoholic extracts from *Thymus pubescens*, *Thymus kotschyanus*, and *Thymus daenensis* (EC_{50} range between 31.47 and 48.68 µg/mL), also six to nine times less active that the positive control, i.e., galic acid [30]; for *T. vulgaris* and wild thyme infusions, the EC_{50} values were 300 and 450 µg/mL, respectively, and corresponded to a 17–25 times lower efficacy than butylated hydroxytoluene [12]. The ability of *Thymus* species to reduce Fe^{3+} to Fe^{2+} is not reported as often; however, Kindl et al. have reported a strong ability for ethanolic extracts from *T. longicaulis*, *T. praecox*, *T. pulegioides*, *T. serpyllum*, *T. striatus*, and *T. vulgaris* (EC_{50} = 11.4–15.1 µg/mL), with the best results being registered for *T. pulegioides*, whose EC_{50} was only 1.7 higher than the reference control [15].

Contrary to the results of the above antioxidant methods, the three *Thymus* extracts presented clear differences in the β-carotene bleaching assay. The oxidation of β-carotene was effectively inhibited by *T. pseudolanuginosus* (EC_{50} = 2.4 ± 0.2 µg/mL), followed by *T. caespititius* (EC_{50} = 6.1 ± 0.2 µg/mL), while *T. herba-barona* was ineffective (Table 1). Notably, literature data also point out promising activity toward the β-carotene oxidation protection from other thymes. In particular, Iauk et al. [29] described an EC_{50} value for a *T. capitatus* methanolic extract that was close to that of 0.7 ± 0.03 µg/mL in

30 min and 1.9 ± 0.6 μg/mL in 60 min. Higher values were observed for the ethanolic extracts from *T. daenensis*, *T. kotschyanus*, and *T. pubescens* (EC_{50} = 23.7, 35.2, and 92.9 μg/mL, respectively) [30].

The combined information from the three antioxidant methods, together with that from the phenolic compounds´ levels, suggests that these last constituents are not the only actors dictating the antioxidant potential of the extracts. Indeed, although phenolic compounds are abundant (particularly flavones) in the *T. pseudolanuginosus* extract, which can be correlated to its superior antioxidant ability in comparison to those of the remaining extracts, the direct phenolic content and antioxidant activity were not observed for those of the two remaining plant species in the β-carotene bleaching assay. This phenomenon could be due to the presence of oxidative—inducer components in the *T. herba-barona* extract, thus mitigating the activity of the antioxidant phenolic compounds.

2.3. Anti-Inflammatory Activity

Because of the major relevance of inflammatory processes in the onset of numerous diseases (cancer, Alzheimer, heart failure, ischemic stroke, and others), the search for low-toxic natural extracts able to counteract pivotal inflammatory players has increased dramatically in the last years [31,32]. The aqueous extracts from the three plant species were screened for their ability to counteract two key inflammatory events, namely the increment of $NO^{\bullet}$ (i.e., a main proinflammatory mediator produced by macrofages) and lipoxygenase (LOX) activity (i.e., the enzyme that controls the production of proinflammatory leukotrienes) [7,33]. As detailed in Table 1, all the extracts revealed a high $NO^{\bullet}$ scavenging ability. Curiously, this effect was more evident for *T. caespititius*, which exhibited the same potency as ascorbic acid (EC_{50} of 229.7 ± 21.5 μg/mL and 228.0 ± 20.7 μg/mL, respectively). Despite having a low ability to inhibit LOX activity, *T. caespetitius* was also the most relevant amongst the three samples (EC_{50} = 590.5 ± 166.3 μg/mL). Hence, the gathered results suggest that *Thymus* aqueous extracts of the selected plants might have potential applications as anti-inflammatory agents, acting through anti-radical capacities towards $NO^{\bullet}$. The fact that this ability is not directly associated the extracts´ phenolic content (*T. caespititius* extract was the less rich in phenolics) also suggests that non-phenolic compounds can have major roles in this action. To our knowledge, the $NO^{\bullet}$ scavenging ability of *Thymus* polar extracts has only been previously reported for ethanolic extracts of six *Thymus* species: *T. serpyllum* subsp. *serpyllum* (EC_{50} = 176.6 ± 8.1 μg/mL), *T. praecox* subsp. *polytrichus* (EC_{50} = 139.0 ± 5.7 μg/mL), *T. vulgaris* (EC_{50} = 97.9 ± 2.9 μg/mL), *T. striatus* (EC_{50} = 91.1 ± 5.3 μg/mL), *T. longicaulis* (EC_{50} = 71.6 ± 4.9 μg/mL), and *T. pulegioides* (EC_{50} = 69.8 ± 4.4 μg/mL) [15], which showed EC_{50} values 1.3–3.2 times higher than the tested standard compound (Trolox), while that of LOX inhibition activity was not exploited.

2.4. Antibacterial Activity

The resistance to antibiotics has been associated with diseases and high mortality rates [34]. Research into new antimicrobial substances, such as purified natural compounds from plants, can serve as one of the therapeutic strategies for the synthesis of new generation and alternative chemical drugs with low toxicity. In this field, phenolic compounds have been gaining magnitude [35]. The antibacterial activity of the Lamiaceae family has been extensively studied, with promising results being observed for *Thymus* essential oils [36,37], while little knowledge has been gathered regarding polar extracts from a thyme origin.

Overall, the collected data showed that, among the five tested strains (Table 3), *Staphylococcus aureus* was the most sensitive to the three *Thymus* extracts, which were able to inhibit both its growth and viability at 0.6, 1.6, and 3.5 mg/mL for *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius*, respectively. These results are consistent with those reported for other *Thymus* species, such as in the study described by Benbelaïd et al. for a water extract of *Thymus lanceolatus*, which exhibited a minimum inhibitory concentration (MIC) value of 1.0 mg/ml for *S. aureus*, as evaluated by the broth microdilution method [22]. Results acquired using the agar dilution method showed MIC values of 0.78 mg/ml for ethanolic extracts of *Thymus caramanicus* when exposed to *S. aureus* [21].

Table 3. MIC (mg/mL) and MBC (mg/mL) of plant extracts and nisin (mg/mL) against selected test pathogens.

Bacteria	*T. herba-barona*		*T. pseudolanuginosus*		*T. caespititius*		Nisin	
	MBC	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**
Salmonella typhimurium	5.0	5.0	>6.5	6.5	>7.0	7.0	0.5	0.5
Staphylococcus epidermidis	5.0	5.0	6.5	6.5	7.0	3.5	<0.03	<0.03
Staphylococcus aureus	0.6	0.6	1.6	1.6	3.5	3.5	0.25	0.25
Escherichia coli	>5.0	5.0	>6.5	6.5	7.0	7.0	0.5	0.5
Pseudomonas aeruginosa	>5.0	5.0	>6.5	6.5	>7.0	7.0	1.0	0.5

Mean values; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

The three *Thymus* plants also exhibited a relevant antibacterial effect against the remaining tested bacteria, although with less potency than the food preservative nisin. In detail, MIC values of 5.0 and 6.5 mg/mL were registered for *T. herba-barona* and *T. pseudolanuginosus*, respectively, against *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Despite *T. caespititius* generally being the less active extract, its activity towards *S. epidermidis* (MIC of 3.5 mg/mL) was more effective than the remaining ones. Note that the MIC concentrations herein found are close to those previously reported for *T. vulgaris* ethanolic extracts, against the same panel of bacteria (MIC in the range 6.25–12.5 mg/mL) [21] or to that of *T. lanceolatus* aqueous extracts against *S. typhimurium* (MIC of 4.0 mg/mL) [22], although they are less effective than a *T. caramanicus* hydroethanolic extract towards *E. coli* and *P. aeruginosa* (MIC values of 1.56 mg/mL and 1.30 mg/mL, respectively) [21].

Overall, the gathered results showed that the thyme extracts were not lethal to some gram-negative bacteria (*S. typhimurium*, *E. coli*, and *P. aeruginosa*), with a high minimum bactericidal concentration (MBC) (Table 3). *T. herba-barona* is effective against *S. typhimurium*, but needs a concentration superior to 5.0 mg/mL to kill *E. coli* and *P. aeruginosa*; a concentration of 6.5 mg/mL of *T. pseudolanuginosus* was not effective against *S. typhimurium*, *E. coli*, and *P. aeruginosa*; *T. caespititius* can be lethal to *E. coli*, but required a concentration superior to 7.0 mg/mL to eliminate *S. typhimurium* and *P. aeruginosa*). Additionally, gram-negative bacteria were more resistant (MIC between 5.0 and 7.0 mg/mL) than gram-positive bacteria, particularly *S. aureus* (MIC in the range 0.6 –3.5 mg/mL). These results are in agreement with those of other authors, who tested aqueous extracts for *T. lanceolatus* (MIC = 4.0 mg/mL against *S. typhimurium* and MIC = 0.83 mg/mL against *S. aureus*) [22]. The same evidence was observed in several researches that tested oils extracted from *Thymus* plants [36,37].

3. Materials and Methods

3.1. Chemicals

Rosmarinic acid, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, eriodictyol-7-*O*-glucoside, luteolin-8-*C*-glucoside, quinic acid, caffeic acid, and *t*-5-*O*-caffeoylquinic acid were obtained from Extrasynthese (Genay Cedex, France). Gallic acid and nisin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent, Na_2CO_3, formic acid, and ethanol were purchased from Panreac (Barcelona, Spain). *n*-hexane, methanol, and acetonitrile with HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). Mueller-Hinton agar was obtained from VWR, Prolabo Chemicals, West Chester, PA, USA. Water was treated in a Direct-Q® water purification system (Merck Life Science, Germany).

3.2. Plant Materials

T. herba-barona, *T. caespititus*, and *T. pseudolanuginosus* species were purchased as a mixture of flowers, leaves, and stems from Ervital (Viseu, Portugal). The plants had been cultivated under an organic regime and, after collection, their aerial parts (flowers, leaves, and stems) were dried in a ventilated incubator at 20–35 °C, for three to five days.

3.3. Extraction of Phenolic Compounds

Phenolic compounds were extracted by decoction, as according to Martins et al. [38], thyme phenolic compounds are efficiently recovered by this methodology. Decoction was performed according to the method described by Ferreira et al. [39], with adaptations. A total of 100 mL of distilled water was added to 5 g of plant material (0.5 mm mesh powder) and the mixture was heated and then boiled for 15 min. After extraction, the mixture was left to stand for 5 min, followed by filtration under reduced pressure through a G3 sintered plates filter. The resulting filtrated solution was concentrated in a rotary evaporator (BUCHI Labortechnik AG, Flawil, Switzerland) at 37 °C, followed by deffating with *n*-hexane (1:1 *v*/*v*). The aqueous defatted fraction was frozen, freeze-dried, and kept under vacuum in a desiccator in the dark, for subsequent use. Three extracts were obtained for each plant.

3.4. Identification and Quantification of Phenolic Compounds

The total phenolic content of each *Thymus* extract was determined according to the adapted Folin-Ciocalteu colorimetric method, as described by Pereira et al. [40]. The individual phenolic compounds were identified by a UHPLC-DAD-ESI-MS^n analysis of extracts (5 mg/mL), performed on Ultimate 3000 (Dionex Co., Sunnyvale, CA, USA) apparatus equipped with an ultimate 3000 Diode Array Detector (Dionex Co.) and coupled to a mass spectrometer. The chromatographic apparatus was composed of a quaternary pump, an autossampler, a photodiode-array detector, and an automatic thermostatic column compartment. The column used had a 100 mm length, 2.1 mm i.d., 1.9 µm particle diameter, and end-capped Hypersil Gold C18 column (Thermo Scientific, Waltham, MA, USA), and its temperature was maintained at 30 °C. Gradient elution was carried out with a mixture of 0.1% (*v*/*v*) of formic acid in water (solvent A) and acetonitrile (solvent B), which were degassed and filtered before use. The solvent gradient consisted of a series of linear gradients, starting with 15–28% of solvent B over 5.6 min, increasing to 29% at 8.8 min, 100% of solvent B at 13.1 min, and maintaining this value up to 17 min, followed by the return to the initial conditions, with a total running time of 20 min. The flow rate used was 0.2 $mL \cdot min^{-1}$ and the UV–Vis spectral data for all peaks were accumulated in the range 200–600 nm. The chromatographic profiles were recorded at 280, 320, and 340 nm.

The mass spectrometer used was a Thermo LTQ XL (Thermo Scientific) ion trap MS equipped with an ESI source. Control and data acquisition were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific). Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with an ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. The full scan covered the mass range from *m/z* 100 to 2000. CID–MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with a collision energy of 25–35 arbitrary units.

For quantitative analysis, the limits of detection (LOD) and quantification (LOQ) were calculated from the parameters of the calibration curves obtained by an injection of known concentrations of the exact or structurally-related standard compounds, represented in Table S1.

3.5. Bioactivity Tests

3.5.1. $DPPH^{\bullet}$ Scavenging Test

The radical scavenging capacity of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* extracts was evaluated by a DPPH radical test, according to the previously described methodology [41]. Ascorbic acid was used as the positive control.

3.5.2. Reducing Power Test

The ability of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* (0.05–0.25 mg/mL) aqueous extracts in reducing iron (III) was assessed by the method previously described [41]. Butylated hydroxyanisole (BHA) was used as the positive control.

3.5.3. β-Carotene Bleaching Carotene

The assay was performed as previously described by Juntachote and Berghofer [42]. A stock emulsion of β-carotene/linoleic acid was initially prepared by dissolving 20 mg of β-carotene in 10 mL of chloroform. A total of 1 mL of the β-carotene solution was added to 1 g of tween 80 and, after chloroform removal, 50 mg of linoleic acid was added. Distilled water (100 mL) was added to the mixture and homogenized using the rotary evaporator. Aliquots of β-carotene/linoleic acid emulsion (250 μL) were mixed with 50 μL of extract at different concentrations and the initial absorbance at 470 nm was immediately recorded. After incubation at 50 °C for 2 h, the reaction was stopped using an ice bath and the absorbance at 470 nm was measured. The blank used was prepared by adding chloroform without β-carotene. BHA was used as the positive control. The % of inhibition was calculated using the formula:

$$\% \text{ of inhibition } = \frac{(C_{t=0} - C_{t=2}) - (E_{t=0} - E_{t=2})}{(C_{t=0} - C_{t=2})} \times 100$$

where $C_{t=0}$ corresponds to the absorbance of control at t = 0 min; $C_{t=2}$ corresponds to the absorbance of control at t = 120 min; $E_{t=0}$, Absorbance of extract at t = 0 min; $E_{t=2}$, Absorbance of extract at t = 120 min.

3.5.3. $NO^{\bullet}$ Scavenging Test

This assay was performed according to the method described by Bor et al. [43]. In brief, 100 μL of sodium nitroprusside (3.33 mM) in PBS 100 mM (pH = 7.4) was added to 100 μL of extract solution at different concentrations (0.07–0.5 mg/mL) and incubated for 15 min at room temperature under light irradiation. The generated $NO^{\bullet}$ interacts with molecular oxygen, producing NO^{2-}, which in the presence of 100 μL of Griess reagent (1% of sulfanilamide and 0.1% of naphthylethylenediamine dihydrochloride in 2.5% of phosphoric acid) produces a purple azo dye. The measurement of the absorbance was determined spectrophotometrically at 562 nm and ascorbic acid was used as the positive control.

3.5.4. Inhibition of 5-Lipoxygenase

The LOX inhibitory assay was performed in a quartz 96-well plate according to the Tappel et al. procedure, with some modifications [44]. During this procedure, 20 μL of the ascorbic acid or extract sample solutions and 20μL of the LOX work solution were added to each well and incubated at 37 °C in the plate reader for 10 min. After incubation, 40 μL of linoleic acid, previously heated at 37 °C, was added and quickly placed in the plate reader. The reaction was followed for 20 min taking measurements every minute at 234 nm. The reaction rate at each inhibitor concentration was calculated by determining the slope of the experimental values and the percentage of inhibition by the following formula:

$$\% \text{ of inhibition } = \frac{v_0 - v_{[inhibitor]}}{v_0} \times 100$$

where v_0 corresponds to the reaction rate of control and $v_{[inhibitor]}$ is the reaction rate of the extract.

3.5.5. Antimicrobial Activity

The antibacterial potential of the *Thymus* polar extracts were evaluated against five bacterial strains, including gram-positive bacteria (*S. epidermidis* NCTC 11047 and *S. aureus* NCTC 6571) and gram-negative bacteria (*S. typhimurium* NCTC 12023, *E. coli* NCTC 9001, and *P. aeruginosa* NCTC 10662) from the National Collection of Type Cultures, operated by Public Health England. All strains were cultured in Mueller-Hinton agar and incubated at 37 °C for 24 h.

The MIC and MBC of plant extracts were determined by the broth microdilution method using a modified standard protocol [45]. Bacterial suspensions were prepared by direct colony suspensions in sterile distilled water and adjusted to obtain 1.5×10^8 colony-forming units (CFU)/mL,

approximately equivalent to 0.5 McFarland units. A final inoculum of 1.5×10^5 CFU/mL was required for suspensions diluted in a 1:100 ratio in Mueller-Hinton broth.

One hundred microliters of Mueller-Hinton broth was dispensed into wells of 96-well micro titer plates (BioTek Instruments, Inc., Winooski, VT, USA). Aqueous solutions of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* extracts were added at a final concentration of 10, 13, and 14 mg/mL, respectively, and were then serially diluted four times across the plate. One hundred microliters of bacteria suspension was finally added to each well and the plates were incubated at 37 °C for 24 h. The assay for each pathogen was repeated three times.

The MIC was defined as the lowest concentration at which visible growth was inhibited while the MBC is the lowest concentration of the tested substance which has a bactericidal effect. MBC values were determined by subculturing 10 µL of the culture from each negative well onto Mueller-Hinton agar and then determining the dilution at which growth was detected [46].

The solvent without extracts served as the negative control and nisin was used as the positive control. Nisin is an antibacterial polypeptide approved as a food preservative whose stock solution was prepared by dissolving 2 mg of nisin in 1 mL of HCl 0.02 N [47].

3.6. Statistical Analysis

All data are presented as mean ± standard deviations. One-way analysis of variance (ANOVA) followed by a Tukey's test were used to detect any significant differences among different means. A *p*-value of less than 0.05 was assumed to indicate a significant difference. The results were analyzed using GraphPad Prism (GraphPad Software, CA, USA, version 6.0).

4. Conclusions

This work elucidates the phenolic composition of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* decoctions, whilst also exploiting their antioxidant, anti-inflammatory, and antimicrobial activities. The three aqueous extracts were mainly characterized by the presence of phenolic acids and flavonoids, which are in part related to their biological activities. *T. pseudolanuginosus* presented the best antioxidant results concerning the three methods used. The selected *Thymus* plants extracts exhibited antibacterial activity against the panel of tested bacteria (*S. typhimurium*, *S. epidermidis*, *S. aureus*, *E. coli*, and *P. aeruginosa*), especially *S. aureus* strains, which were in general the most sensitive. *T. caespititius* appears to have anti-inflammatory potential, based on its promising inhibitory activity on $NO^\bullet$ production. Bearing in mind the increasing demand for bioactive extracts of a botanical origin, this work opens a door for the expansion of the commercial exploitation of the thyme species.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/18/9/1879/s1.

Acknowledgments: The authors acknowledge to the Foundation for Science and Technology (FCT), the European Union, the National Strategic Reference Framework (QREN), the European Regional Development Fund (FEDER), and Operational Programme Competitiveness Factors (COMPETE), for funding the Organic Chemistry Research Unit (QOPNA) (project PEst-C/QUI/UI0062/2013; FCOMP-01-0124-FEDER-037296).

Author Contributions: Andrea F. Afonso and Olívia R. Pereira performed the experimental work, the analysis, and the interpretation of data, and wrote the paper; Rodrigo T. Neto contributed to some antioxidant assays; Artur M.S. Silva co-wrote the manuscript; Susana M. Cardoso coordinated the work and co-wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Metabolitos y actividades biológicas de *Thymus zygis, Thymus pulegioides* y *Thymus fragrantissimus* obtenidos por cultivo orgánico

Objetivos

El presente estudio tiene como objetivo evaluar la composición nutricional de tres especies de timo económicamente importantes (*Thymus zygis, Thymus pulegioides* y *Thymus fragrantissimus*) cultivadas en un régimen orgánico, así como establecer el perfil fenólico y las actividades biológicas (antioxidante y antimicrobiana) de sus respectivas decocciones.

1. Introducción

Los productos medicinales y aromáticos cultivados orgánicamente son más fácilmente aceptados por los mercados globales [1], y se consideran más saludables por los consumidores, ya que esta práctica agrícola restringe el uso de fertilizantes sintéticos, pesticidas y herbicidas [2,3]. En este contexto, en varios estudios [4] se encontró que las concentraciones de una gama de polifenoles como los ácidos fenólicos, flavanonas, estilbenos, flavonas, flavonoles y antocianinas estaban sustancialmente aumentadas en alimentos de cultivos orgánicos. Las plantas de tomillo cultivadas pueden comercializarse para una amplia gama de aplicaciones, especialmente en la cocina [5,6] y en la medicina tradicional [7]. La aplicación generalizada de las plantas *Thymus* ha estado asociada durante mucho tiempo con su agradable sabor y olor, pero en la actualidad, los componentes de estas plantas se han asociados estrechamente con su alto valor nutricional debido a los compuestos bioactivos, como sus aceites esenciales pero también, con creciente interés, sus extractos polares [8,9].

Por lo que sabemos, este es el primer trabajo que se centra en las plantas *T. zygis, T. pulegioides* y *T. fragrantissimus* cultivadas orgánicamente. También hay que señalar que la composición química y las potencialidades biológicas de estas especies botánicas han sido poco estudiadas, incluso para las plantas silvestres [10,11].

2. Métodos

2.1. Plantas y extracción de compuestos fenólicos

Las especies *T. zygis, T. fragantissimus* y *T. pulegioides* han sido compradas a la empresa Ervital (Viseu) donde se cultivaron bajo un régimen orgánico y en que sus partes aéreas (flores, hojas y tallos) fueron recogidas y secas. No se aplicó ningún tratamiento químico o bacteriológico a las plantas. Su apariencia y comportamiento

fueron comprobados regularmente para asegurar su salud y la ausencia de patógenos. Cuando fue apropiado, las malas hierbas fueron eliminadas del suelo circundante.
Los extractos fenólicos de los tres tomillos se han obtenido utilizando una decocción en la proporción 1:20 (5 g en 100 ml de agua). Las soluciones se filtraron, se concentraron y el concentrado se desengrasó con *n*-hexano (1:1 v/v), se evaporó y se congeló a -20°C y se liofilizó. Se obtuvieron tres extractos para cada planta.

1.2 Análisis nutricional

El contenido de proteína bruta de las muestras se determinó mediante el método macro-Kjeldahl. El contenido de ceniza y de la grasa cruda se determinaron por cuantificación gravimétrica. Los carbohidratos totales se calcularon por diferencia. El contenido de calorías se calculó de acuerdo con la ecuación: Energía (kcal) = 4 × (g de proteína + g de carbohidratos) + 9 × (g de grasa) [10].
Los minerales (Na, K, Ca, Mg, Fe, Mn, Cu y Zn) se cuantificaron mediante espectrometría de absorción atómica de llama. Los ácidos grasos fueron analizados por un método de cromatografía de gases.

2.3 Identificación y cuantificación de compuestos fenólicos

El contenido fenólico total de cada extracto de tomillo se determinó de acuerdo con el método colorimétrico de Folin-Ciocalteu, cuyos valores se expresaron en equivalentes de ácido gálico (EAG)/g de extracto. Los compuestos fenólicos individuales fueron caracterizados y cuantificados mediante cromatografía líquida de alta eficiencia (UHPLC-DAD-ESI-MS^n).

2.4 Ensayos de bioactividad

2.4.1 Capacidad antioxidante

La actividad antioxidante de los extractos acuosos de tomillo se evaluó mediante dos ensayos químicos: i) prueba de radicales 2,2-difenil-1-picrilhidrazilo ($DPPH^{\bullet}$), usando ácido ascórbico como control positivo; ii) el cálculo del poder reductor por la reducción de hierro (III), usando hidroxianisol butilado (BHA) como estándar. Los resultados se expresaron como valores de EC_{50} (concentración de la muestra que proporciona el 50% de la actividad antioxidante) en µg por ml de extracto.

2.4.2 Capacidad antibacteriana

El potencial antibacteriano de los extractos polares de *Thymus* se evaluó frente a cinco cepas bacterianas, incluyendo dos Gram positivas (Gram+) (*Staphylococcus epidermidis* y *Staphylococcus aureus*) y tres Gram negativas (Gram-) (*Salmonella typhimurium, Escherichia coli y Pseudomonas aeruginosa*), por el método de microdilución en medio, para calcular la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM). Se utilizó la nisina como compuesto de referencia.

3. Resultados y Discusión

3.1. Composición nutricional

El perfil nutricional de las tres especies de *Thymus* se muestra en la Tabla 1. Las plantas secas eran esencialmente ricas en carbohidratos, variando de 78 a 87% peso en seco (ps), mientras que los contenidos en proteína, grasa y ceniza estaban en los rangos de 3.6–10.5%, 2.3–4.5% y 5.0–8.2%, respectivamente. *T. pulegioides* fue la especie más rica con respecto a los niveles de estos macronutrientes en comparación con los resultados reportados por Fernandes et al. [10] para *T. pulegioides* silvestre recolectados en el noreste de Portugal.

T. pulegioides fue también la especie más rica con respecto a su contenido mineral. Sin embargo, hay que subrayar que las tres plantas presentaron niveles altos de Ca (0.57–1.04 g/100 g ps) y K^+ (1.23–2.19 g/100 g ps), pero cantidades moderadas de Na^+, lo que representa altos niveles de provisión de K^+/Na^+, que es de gran importancia cuando se trata de compensar la dieta occidental moderna, que suele ser rica en NaCl. Esta relación fue particularmente evidente en *T. pulegioides*, debido a su riqueza en K^+ (K^+/Na^+=294), un hecho que determina un enorme potencial de la planta para su aplicación en alimentos funcionales dirigidos a salud cardiovascular.

Con respecto a los ácidos grasos, los resultados indicaron que las plantas de tomillo eran más ricas en ácidos grasos insaturados (AGI), que representaban del 69 al 74%, mientras que los ácidos grasos saturados (AGS) representaban del 27 al 31% del total de los ácidos grasos. Entre los AGI, los ácidos grasos poliinsaturados (AGPI) fueron los principales representantes, y oscilaron entre 48, 49 y 55% en *T. zygis*, *T. pulegioides* y *T. fragrantissimus*, respectivamente.

En general, el AGPI comprendía ácido linoleico (C18: 2n6c) y ácido α-linolénico (C18: 3n3), mientras que la fracción de ácidos grasos monoinsaturados (AGMI) estaba compuesta de ácido oleico (C18: 1n9c) y ácido erúcico (C22: 1n9). Aunque encontramos varios AGS, el ácido palmítico (C16: 0) fue el más abundante en las tres muestras, ya que representó entre el 18 y el 24% de los ácidos grasos. Debido a eso, la proporción de AGPI/AGS de las plantas de tomillo fue considerablemente alta (1.62-1.83). Esto es particularmente relevante teniendo en cuenta la recomendación de la Organización Mundial de la Salud para el consumo de alimentos ricos en AGPI como parte de un estilo de vida saludable, orientado a la protección cardiovascular [12,13].

Además, las proporciones de ácidos grasos omega-6/omega-3 se encuentran dentro de los valores recomendados (<10) [14], y las especies *T. fragrantissimus* y *T. pulegioides* (0.54 y 0.75, respectivamente) asumen relevancia debido a su bajo valor, que se cree

que está asociado con la prevención de muchas enfermedades inflamatorias crónicas, incluyendo la artritis reumatoide, la inflamación intestinal y varias otras condiciones [13,15].

Tabla 1. Valor nutricional de las tres especies de plantas de *Thymus*

Contenido total	***T. zygis***	***T. pulegiodes***	***T. fragrantissimus***
Carbohydratos total (g/100 g ps)	86.88 ± 0.51	81.22 ± 0.90	77.56 ± 0.16
Proteínas (g/100 g ps)	3.59 ± 0.13	8.28 ± 0.81	10.49 ± 0.13
Ceniza (g/100 g ps)	5.02 ± 0.25	8.24 ± 0.06	7.66 ± 0.22
Minerales (mg/100 g ps) *			
Na	7.6	7.4	9.0
K	1229.3	2185.6	1719.5
Ca	566.0	1039.4	865.6
Mg	119.8	148.3	241.8
Fe	4.4	1.9	7.8
Mn	9.8	15.7	6.9
Cu	0.1	0.1	0.3
Zn	2.1	1.7	2.7
Grasa (g/100 g ps)	4.52 ± 0.12	2.27 ± 0.03	4.30 ±0.19
Ácidos grasos (relativo %) *			
C12: 0	0.48	0.5	0.7
C14: 0	1.2	1.1	1.4
C16: 0	18.8	23.6	23.3
C18: 0	3.2	3.3	2.8
C18: 1n9	16.9	19.0	8.6
C18: 2n6c	32.6	20.4	19.3
C18: 3n3	15.6	27.6	36.2
C20: 0	2.8	1.8	2.6
C22: 1n9	8.3	2.7	5.0
AGS	26.5	30.2	30.9
AGMI	25.3	20.9	13.8
AGPI	48.3	48.9	55.3
AGPI/AGS	1.8	1.6	1.8
n-6/n-3	2.1	0.8	0.5
Contenido calórico (kcal/100 g ps)	402.50 ± 0.42	378.39 ± 0.08	390.86 ± 1.83

AGS: ácidos grasos saturados; AGMI: ácidos grasos monoinsaturados; AGPI: ácidos grasos poliinsaturados; Ácido laurico (C12: 0); Ácido mirístico (C14: 0); Ácido palmítico (C16: 0); Ácido esteárico (C18: 0); Ácido oleico (C18: 1n9c + t); Ácido linoleico (C18: 2n6c); Ácido α-linolénico (C18: 3n3); Ácido araquidico (C20: 0); Ácido erúcico (C22: 1n9); * S.E.M. < 10%.

Los datos presentados en este documento apuntan a una menor prevalencia de AGS y mayores cantidades de AGMI en comparación con estudios anteriores, particularmente en *T. pulegioides* silvestre [10], cuyas diferencias pueden estar naturalmente

relacionadas con múltiples factores, incluidas las condiciones de crecimiento (agricultura orgánica *vs* silvestre).

3.2. Caracterización fenólica de las decocciones de tomillo

El rendimiento de los extractos de decocción de las tres especies de *Thymus* ha sido 12%, 16% y 25% en *T. zygis, T. fragrantissimus* y *T. pulegioides*, respectivamente. La decocción de *T. pulegioides* también mostró un contenido superior de compuestos fenólicos totales en comparación con *T. zygis* y *T. fragrantissimus* (391, 288 y 287 µg EAG/mg de extracto, respectivamente).

Los constituyentes fenólicos individuales de los tres extractos acuosos de tomillo fueron determinados mediante UHPLC-DAD-ESI-MS^n (Tabla 2). Estos eran principalmente ricos en ácido rosmarínico (fracción 26), consistente con estudios previos sobre extractos polares de tomillo [16,17]. Curiosamente, sus niveles (62–82 µg/mg de extracto) fueron más altos que los encontrados previamente para las decocciones de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius* (40–56 µg/mg extracto) [17], así como para extractos hidroetanólicos de *Thymus* x *citriodorus* (10 µg/mg de extracto) [18] y extractos metanólicos de *Thymus praecox* (15 mg/g de peso seco (ps)) [19]. Se encontró la misma tendencia para los isómeros del cafeoil- ácido rosmarínico (MW 538, eluidos en las fracciones 27, 29, 30, 31 y 32), que ascendieron de 16 a 60 µg/mg de extracto en estas especies. El ácido cafeico y sus derivados (ácido rosmarínico, cafeoil ácido rosmarínico, ácido salvianólico I/H) representaron la mayoría de los componentes fenólicos en los extractos acuosos de *Thymus* (valores de p <0,01), aunque fueron menos representativos en *T. pulegioides* (64% de compuestos fenólicos totales) en comparación con las especies restantes (cerca del 80%).

Tabla 2. Identificación y cuantificación de compuestos en extractos de *T. zygis*, *T. pulegioides* y *T. fragrantissimus* por UHPLC-DAD-ESI-MS^n

Fr	TR (min)	λmax	Compuesto	$[M-H]^-$	*T. zig*	*T. pul*	*T. fragr*
1	1.3	298	Ácido quinico	191	D	D	D
2	1.6	277	Ácido siringico hexósido	359	D	D	D
3	1.8	281	Danshensu	197	D	D	D
4	1.9	283	Eriodictiol- di-*O*-hexósido	611	-	D	-
5	2.4	271, 326	Apigenina di-*C*- glucósido	593	1.60 ± 0.32^a	-	2.69 ± 0.50^a
6	3.3	283	Eriodictiol-*O*-hexosido (isómero 1)	449	-	9.43 ± 0.21	-
7		289, 321	Ácido cafeico	179	8.46 ± 0.02^a		2.54 ± 0.14^b

8	3.4	285	Derivado del ácido salvianólico F	375	D	-	D
9	3.7	283	Eriodictiol-*O*-hexosido (isómero 2)	449	6.02 ± 0.94[a]	9.90 ± 0.19[b]	2.71 ± 0.03[c]
10	4.1	281, 342	Quercetina glucurónido	477	-	3.28 ± 0.03[a]	1.13 ± 0.25[b]
11	4.2	282, 342	Quercetina-*O*-hexosido	463	2.97 ± 0.90[a]	-	1.48 ± 0.02[b]
12	4.6	341	Luteolina-*C*-glucósido	447	4.86 ± 0.03[a]	8.27±0.13[b]	2.00 ± 0.02[c]
13	5.6	281, 342	Ácido salvianólico I/H	537	-	D	-
14	6.2	258, 268, 342	Luteolina-*O*-di glucósido	593	1.19 ± 0.01[a]	-	0.36 ± 0.02[b]
15	6.2	283	Naringenina-*O*-glucósido	433	-	1.90 ± 0.97	-
16	6.8	282, 333	Scutelarina-*O*-glucurónido	461	D	D	-
17	7.0	254, 265, 345	Luteolina-*O*-glucurónido (isom1)	461	7.57 ± 0.05[a]	26.14 ± 0.78 [b]	16.86 ± 0.21[c]
18	7.1	283	Eriodictiol-*O*-glucurónido	463	-	D	D
19	7.3	282, 336	Quercetina-*O*-pentosido	433	D	-	-
20	7.6	288, 321	Ácido rosmarínico hexosido	521	D	-	D
21		254, 267, 344	Luteolina-*O*-glucurónido (isómero 2)	461	-	D	-
22	8.5	240, 339	Crioseriol-*O*-hexosido	461	0.78 ± 0.01[a]	12.00 ± 0.15[b]	
23		281	Derivado del ácido cafeico	553	-	-	D
24	8.7	283	Ácido sagerínico	719	D	D	D
25	9.3	267, 334	Apigenina-O-glucurónido	445	D	9.20 ± 0.21[a]	1.76 ± 0.10[b]
26	9.6	290, 328	Ácido rosmarínico	359	62.36 ± 2.72[a]	81.65 ± 7.02[b]	81.04 ± 7.93[b]
27	9.8	287, 311	Cafeoil ácido rosmarínico (isómero 1)	537	2.79 ± 0.24[a]	5.25 ± 0.19[b]	-
28	10.1	287, 328	Ácido salvianólico B	717	1.89 ± 0.20	-	-
29	10.7	290, 323	Cafeoil ácido rosmarínico (isómero 2)	537	19.40 ± 0.76[a]	57.73 ± 1.95[b]	16.21 ± 0.54[c]
30	12.1	288, 322	Cafeoil ácido rosmarínico (isómero 3)	537	D	D	D
31	12.5	287, 328	Cafeoil ácido rosmarínico (isómero 4)	537	-	D	-
32	12.8	288, 323	Cafeoil ácido rosmarínico (isómero 5)	537	-	D	-
			Total		119.9 ± 3.31[a]	225.79 ± 15.08[b]	126.65 ± 9.73[a]
			Ácido cafeico y derivativados		94.89 ± 1.84[a]	144.63 ± 8.79[b]	101.12 ± 6.75[a]
			Flavonas		16.01 ± 0.27[a]	55.62 ± 1.05[b]	21.67 ± 0.59[c]
			Flavonoles		2.97 ± 0.90[a]	3.28 ± 0.03[a]	2.62 ± 0.25[a]
			Flavanonas		6.02 ± 0.94[a]	19.93 ± 0.46[b]	2.71 ± 0.03[c]

D: Detectado; Fr: fracción; TR: tiempo de retención; *T. zig-T. zygis; T. pul-T. pulegioides*; *T. fragr-T. fragrantissimus*; Valores medios ± SD de tres ensayos independientes; El análisis estadístico fue realizado por one-way ANOVA (prueba de Tukey. deTukey). En cada fila diferentes letras significan diferencias estadísticamente significativas ($p < 0.05$). Los valores se expresan como μg/mg.

A su vez, la decocción de *T. pulegioides* fue la más rica en flavonas (55.62 ± 1.05 µg/mg de extracto, $p < 0.001$), representada principalmente por luteolina-*O*-glucurónido (fracciones 17 y 21), luteolina-*C*-glucósido, crioseriol-*O*-hexósido y apigenina-*O*-glucurónido (fracciones 12, 22 y 25, respetivamente). En general, los niveles de flavonas en la decocción de *T. pulegioides* fueron más altos que los encontrados previamente en *T. herba-barona* y *T. caespititius* (22 y 32 µg/mg de extracto), pero más bajos que *T. pseudolanuginosus* (73 µg/mg de extracto) [17].

El extracto de *T. pulegioides* también presentó mayores cantidades de flavanonas (19.93±0.46 µg/mg de extracto), principalmente compuesto por eriodictyol-*O*- hexósido y naringenina-*O*-glucósido. Se señala que los compuestos descritos aquí (derivados del ácido cafeico y flavonoides, incluyendo las formas glicosídicas) se detectaron previamente en extractos polares de otras especies de *Thymus* [16,18].

3.3 Actividad antioxidante

En este estudio, la capacidad antioxidante de las decocciones de *T. zygis, T. fragrantissimus* y *T. pulegioides* se analizó mediante dos ensayos, a saber, el método de captación de $DPPH^{\bullet}$ y del poder reductor, que evalúan respectivamente la capacidad para atrapar los radicales libres sintéticos DPPH y la capacidad de reducir el ion férrico (Fe^{3+}) a ion ferroso (Fe^{2+}), respectivamente. En general, los extractos presentaron una alta capacidad antioxidante, ya que los valores de EC_{50} eran hasta dos veces más altos que los compuestos de referencia comerciales (Tabla 3). Entre los tres extractos, *T. pulegioides* tuvo el valor más bajo de $DPPH^{\bullet}$ EC_{50} (9.50 µg/mL), alrededor de 1.4 veces el ácido ascórbico (EC_{50}=6.9µg/mL), mientras que *T. fragrantissimus* y *T. zygis* se establecieron a 13 µg/mL. Este hecho muestra la mayor capacidad de *T. pulegioides* para eliminar el radical libre $DPPH^{\bullet}$ en comparación con las otras dos especies de plantas y posiblemente puede asociarse con su mayor contenido fenólico. Relativamente al poder reductor (EC_{50}=30.73 µg/mL), la superioridad antioxidante de *T. pulegioides* no fue tan clara, lo que sugiere que los componentes no fenólicos también podrían desempeñar un papel relevante en esta reacción.

La alta capacidad antioxidante aquí descrita para las decocciones de *T. pulegioides* superó la descrita anteriormente para el extracto metanólico de *T. pulegioides* silvestre (EC_{50}=680 y 490 µg/mL para $DPPH^{\bullet}$ y poder reductor, respectivamente) [10]. Más recientemente, Kindl et al. [11] encontraron resultados prometedores para un extracto hidroananólico de *T. pulegioides* silvestre, (EC_{50}=4.18 y 11.39 µg/mL en las pruebas $DPPH^{\bullet}$ y poder reductor, respectivamente), que correspondió a aproximadamente 1.7-2.5 veces de los compuestos comerciales de referencia. Sin embargo, según nuestro conocimiento, no hay datos bibliográficos sobre la capacidad antioxidante de extractos

acuosos de *T. zygis, T. pulegioides* o *T. fragrantissimus.* Aun así, las altas habilidades reportadas en el presente documento para las decocciones están en línea con las descritas anteriormente por nuestro grupo para extractos acuosos de *T. herba-barona, T. pseudolanuginosus* y *T. caespititius* [17].

Tabla 3. Actividad antioxidante y antibacteriana de extractos acuosos de tres plantas de tomillo

	T. zygis	***T. pulegioides***	***T. fragrantissimus***	**Estándar**
Actividad antioxidante				
DPPH• (1) (μg/mL)	12.65 ± 2.30 ^{a,b}	9.50 ± 1.98 ^{a,b}	12.87 ± 3.79 ^{a}	6.90 ± 0.5 ^{b}
Poder reductor (2) (μg/mL)	33.66 ± 1.93 ^{a}	30.73 ± 1.48 ^{a}	32.44 ± 4.27 ^{a}	16.30 ± 1.5 ^{b}
Actividad antibacteriana (3) (CIM/CBM, mg/mL)				
G (+) bacteria				
S. aureus	1.13/1.13	5.75/5.75	3.75/3.75	0.25/0.25
S. epidermidis	4.50/4.50	5.75/11.50	7.50/>7.50	<0.06/<0.06
G (-) bacteria				
S. typhimurium	4.50/4.50	5.75/11.50	7.50/7.50	0.50/0.50
E. coli	>4.5/>4.5	>11.50/>11.50	7.50/>7.50	0.50/0.50
P. aeruginosa	4.5/>4.5	5.75/11.50	7.50/>7.50	0.50/1.0

(1) Cantidad de extracto requerido para reducir 50% de 60 μM DPPH•; Se utilizó ácido ascórbico como compuesto de referencia; (2) Cantidad de extracto capaz de proporcionar 0.5 de absorbancia reduciendo 3.5 μM Fe^{3+} a Fe^{2+}; Se utilizó BHA como compuesto de referencia; Valores medios ± SD; El análisis estadístico se realizó mediante ANOVA de una vía, seguido de la prueba de Tukey. En cada fila, letras diferentes (a, b) significan diferencias estadísticamente significativas ($p < 0.05$); (3) La nisina se utilizó como compuesto de referencia; Valores medios; CIM: Concentración inhibitoria mínima; CBM: concentración bactericida mínima.

3.4. Actividad antibacteriana

En el presente estudio, evaluamos la actividad inhibitoria y letal de los extractos acuosos de *T. zygis, T. pulegioides* y *T. fragrantissimus* contra cinco bacterias, a través del ensayo de microdilución en medio. *S. aureus* fue la especie más sensible a las decocciones del tomillo, y su crecimiento y viabilidad se inhibieron a 1.13, 3.75 y 5.75 mg/ml para *T. zygis, T. fragrantissimus y T. pulegioides*, respectivamente (Tabla 3). Los resultados, combinados con los informados previamente para T*. herba-barona, T. pseudolanuginosus* y *T. caespititius* [17], nos permiten concluir que, entre los seis extractos acuosos de tomillo, *T. herba-barona* y *T. zygis* son los dos más activos contra *S. aureus* (CIM/CBM de 0.6 y 1.13 mg/ml, respectivamente). Entre las bacterias Gram negativas, *S. typhimurium* fue la más susceptible, y se ha inhibido tanto su crecimiento como su viabilidad frente a *T. zygis, T. fragrantissimus* y *T. pulegioides* (concentraciones inhibitorias y/o letales en el intervalo 4.50-11.5 mg/ml). Se informaron valores de la

misma magnitud cuando *S. typhimurium* fue tratado con extractos polares de *Thymus lanceolatus* [20], *Thymus capitatus* [21], *Thymus caramanicus* y *Thymus vulgaris* [22], presentando valores CIM de 4.0, 6.0, 2.6 y 12.5 mg/ml, respectivamente.
A su vez, *P. aeruginosa* y *E. coli* fueron incluso más resistentes a los extractos de tomillo, aunque a diferentes niveles. Si bien el crecimiento de *P. aeruginosa* fue inhibido por todas las muestras (Tabla 3), solo *T. fragrantissimus* tuvo un impacto negativo en el crecimiento de *E. coli* a 7.5 mg/ml. Además, *T. pulegioides* fue capaz de matar a *P. aeruginosa* (CBM=11.5 mg/ml), mientras que no se observó ningún efecto sobre la viabilidad de *E. coli*. Se ha demostrado previamente que extractos polares de plantas de tomillo han inhibido cepas de *P. aeruginosa* (CIM de 0.25 y 2.0 mg/ml) [20,23], y *E. coli* (MIC en el rango de 0.5–8 mg/ml) [22,23], aunque no se obtuvo información sobre sus efectos sobre la viabilidad de estas bacterias.

4. Conclusiones

La composición nutricional, el perfil fenólico, las actividades antioxidantes y antimicrobianas de tres especies de timo cultivadas en la agricultura orgánica se informan en este estudio. Con base en nuestros resultados, es posible inferir que estas especies de tomillo presentan una riqueza de ácidos grasos poliinsaturados y una interesante relación de ácidos grasos omega-6/omega-3, junto con altas proporciones de K^+/Na^+ que pueden contribuir a la protección cardiovascular.
T. pulegioides presentó la mayor capacidad antioxidante, en consonancia con su alto contenido en polifenoles, en los que se destacan el ácido rosmarínico y el cafeoil ácido rosmarínico, junto con niveles considerables de flavonas y flavanonas. Además, los extractos acuosos obtenidos de estas especies de tomillo, también mostraron potencial antibacteriano.

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Article

Metabolites and Biological Activities of *Thymus zygis*, *Thymus pulegioides*, and *Thymus fragrantissimus* Grown under Organic Cultivation

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Received: 19 May 2018; Accepted: 18 June 2018; Published: 22 June 2018

Abstract: *Thymus* plants are marketed for diverse usages because of their pleasant odor, as well as high nutritional value and wealth of health-promoting phytochemicals. In this study, *Thymus zygis*, *Thymus pulegioides*, and *Thymus fragrantissimus* grown under organic cultivation regime were characterized regarding nutrients and phenolic compounds. In addition, the antioxidant and antibacterial properties of these species were screened. The plants were particularly notable for their high K/Na ratio, polyunsaturated fatty acids content and low omega-6/omega-3 fatty acids ratios, which are valuable features of a healthy diet. Caffeic acid and/or its derivatives, mainly rosmarinic acid and caffeoyl rosmarinic acid, represented the majority of the phenolic constituents of these plants, although they were less representative in *T. pulegioides*, which in turn was the richest in flavones. The latter species also exhibited the highest antioxidant capacity (DPPH$^{\bullet}$ EC_{50} of 9.50 ± 1.98 μg/mL and reducing power EC_{50} of 30.73 ± 1.48 μg/mL), while *T. zygis* was the most active towards Gram-positive and Gram-negative bacteria. Overall, the results suggest that the three thyme plants grown in organic farming are endowed with valuable metabolites that give them high commercial value for applications in different industries.

Keywords: *Thymus*; thyme; phenolic compounds; nutrients; antioxidant; antibacterial activity

1. Introduction

Thymus plants, belonging to the Lamiaceae family, represent a large botanical genus well known in the West Mediterranean region. Due to their high capacity to adapt to extreme climate conditions concerning temperature and water supply, the plants can often be found in rocks or stones growing in cold and arid conditions [1]. In general, they appear as perennial and subshrubs or shrubs 10 to 30 cm tall, with small and simple leaves, a quadrangular stem erect to prostate, ramified and prostrated branches, and big clusters of small pink, white, cream or purple flowers [2,3].

Because of its easy growth and the wide variety of *Thymus*-derived products that can be used by diverse industries, the cultivation of thyme species for commercial purposes has increased greatly in recent decades [4]. Special interest is given to organic farming, since this is recognized as a sustainable agricultural system. Indeed, organic agriculture has developed rapidly worldwide in recent years,

being presently practiced in approximately 120 countries. At the level of the European Union, the area under organic production increased by about 70% in the last decade, reaching about 11 million hectares [5]. Moreover, medicinal and aromatic plants represent one of the top 10 crops cultivated under the organic regime. Organically grown medicinal and aromatic products are readily accepted in global markets and command higher prices than those grown with chemical inputs [6]. Indeed, crops grown under an organic regime are considered healthier by consumers, since this agriculture practice restricts the use of synthetic fertilizers, pesticides, and herbicides [7,8]. Moreover, there is evidence that organic agriculture can lead to more valuable products. In this context, the concentrations of a range of polyphenolics such as phenolic acids, flavanones, stilbenes, flavones, flavonols, and anthocyanins were found to be substantially higher in organic crops/crop-based foods in several studies [9]. In particular, Sousa et al. [10] showed that the levels of phenolic compounds in leaves of tronchuda cabbage from organic culture had higher amounts of phenolics, probably due to the interference of mineral fertilizers and pesticides with the biosynthetic pathway of phenolic compounds.

Cultivated *Thymus* plants can be commercialized for a wide range of applications. In fact, *Thymus* are amongst the main aromatic plants used as culinary ingredients for fish, meat, salad and vegetable dishes [11,12]. Also, dried thyme leaves are used for teas as well as in other several products such as lotion, bath soap, and toothpaste [1,13]. In addition, thyme-derived products are applied in traditional medicine to treat respiratory and throat ailments, and skin problems such as oily skin, sciatica, acne, dermatitis, parasite affections, eczema, fungal infections, and insect bites, among others [14]. Moreover, thyme essential oil is a globally respected commodity and is currently used in many industries such as food, pharmaceuticals, personal health care, detergents, and insecticides [1,15].

The widespread application of *Thymus* plants has long been associated with their pleasant taste/odor, but at present, highly valuable products of these plants are closely associated with their high nutritional value and/or predominance of bioactive compounds. Among the latter, essential oils have undoubtedly been the most exploited health promoters of *Thymus*, being recognized for their antimicrobial, preservative, antispasmodic, diuretic, antihypertensive, and calming properties [12,16,17]. Although less studied, a growing number of investigations have been focused on the potential biological activities of extracts rich in phenolic compounds, and their prospective for application as high-value products [16,17].

The present study aims to evaluate the nutritional composition of three economically important *Thymus* species (*Thymus zygis*, *Thymus pulegioides*, and *Thymus fragrantissimus*) (Figure S1) cultivated in an organic farming regime, as well as to establish the phenolic profile and biological potencies (antioxidant and antimicrobial) of their respective decoctions. As far as we know, this is the first work focusing on organically grown *T. zygis*, *T. pulegioides*, and *T. fragrantissimus* plants. One must also remark that the chemical composition and/or biological potentials of these botanical species remain poorly studied, even for wild plants. In this regard, Fernandes et al. [18] previously reported the nutritional value of a wild *T. pulegioides* collected in the northeast of Portugal. In addition, these authors and Kindl et al. [19], also evaluated the antioxidant potential of methanolic or hydroethanolic extracts of wild *T. pulegioides* (in northeast Portugal and Croatia) against oxidative events.

2. Results and Discussion

2.1. Nutritional Composition

The nutritional profile of the three *Thymus* species cultivated under an organic farming regime is shown in Table 1. The dried plants were essentially rich in carbohydrates, varying from 78 to 87% dw, while protein, fat, and ash contents were in the ranges of 3.6–10.5%, 2.3–4.5%, and 5.0–8.2%, respectively. The levels of these macronutrients in *T. pulegioides* were 81.2, 8.3, 2.3, and 8.2 g/100 g dw, respectively, which, compared to those reported by Fernandes et al. [18] for a wild *T. pulegioides* collected in the northeast of Portugal (89.4, 5.5, 0.2, and 4.9 g/100 g dw, respectively), presented higher

non-carbohydrate macronutrients. To our knowledge, there are no previous data on the nutritional value of *T. zygis* and *T. fragrantissimus* plants.

T. pulegioides was also the richest species with respect to its mineral content. However, it is worth mentioning that all three plants had high levels of Ca (0.57–1.04 g/100 g dw) and K (1.23–2.19 g/100 g dw), but modest Na amounts, thus representing high K/Na suppliers, which is of great importance when aiming to compensate for the modern Western diet, which is typically rich in NaCl. This ratio was particularly high in *T. pulegioides*, which, due to its richness in K, was established at 294, a fact that can give the plant enormous potential for application in functional foods directed to cardiovascular health claims.

Regarding fatty acids, the results indicated that thyme plants were richer in unsaturated fatty acids (UFA), which represented 69–74%, whereas saturated fatty acids (SFA) represented 14 to 25% of the total fatty acids. Among UFA, polyunsaturated fatty acids (PUFA) were the main representatives, ranging from 48 to 49% in *T. zygis* and *T. pulegioides*, to 55% in *T. fragrantissimus*. Overall, PUFA comprised linoleic acid (C18: 2n6c) and α-linolenic acid (C18: 3n3), while monounsaturated fatty acids (MUFA) fraction was composed of oleic acid (C18: 1n9c) and erucic acid (C22: 1n9) (structures in Figure 1).

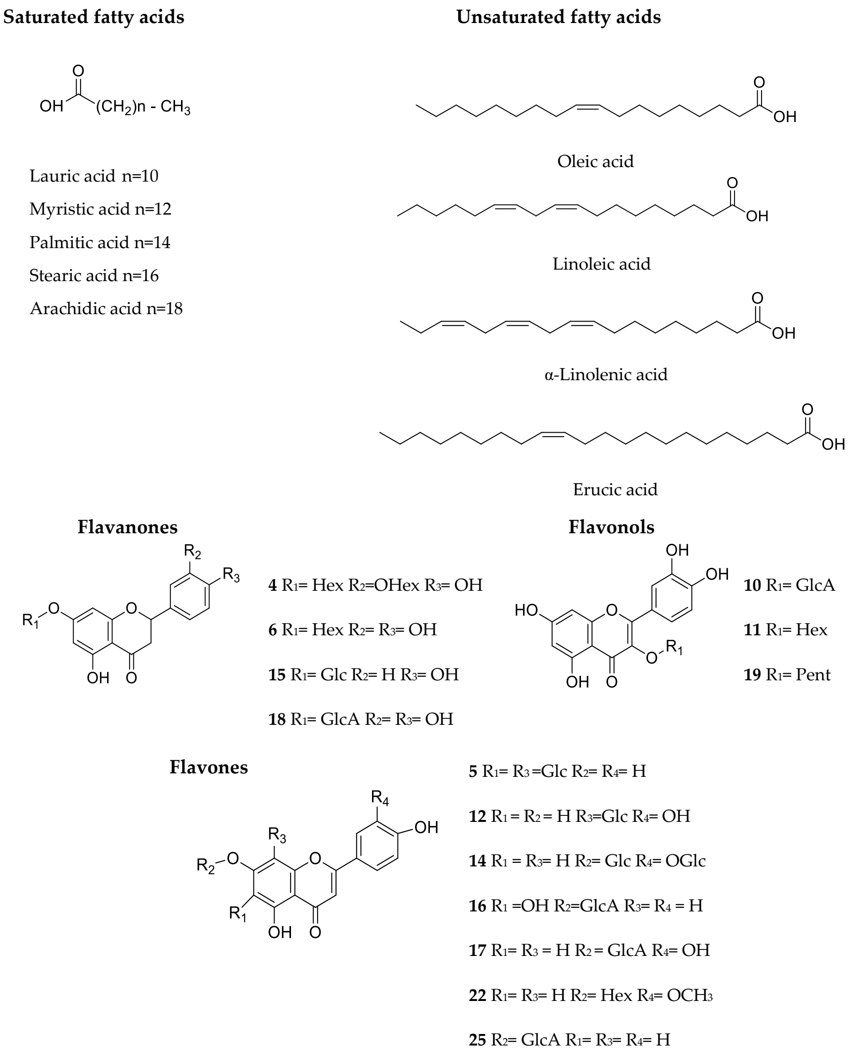


Figure 1. *Cont.*

Figure 1. Chemical structures of fatty acids and phenolic compounds identified in *Thymus zygis*, *Thymus pulegioides*, and *Thymus fragrantissimus*. Numbers in the figure correspond to the UHPLC-DAD-ESI-MS^n peaks of Figure 2.

Although we found several SFA, palmitic acid (C16: 0) was by far the most abundant in the three samples, accounting for 18–24% of the fatty acids. Because of that, the PUFA/SFA ratio of the thyme plants was considerably high (1.62–1.83). This is particularly relevant when one examines the recommendation of the World Health Organization for the consumption of PUFA-rich foods as part of a healthy lifestyle, aiming at cardiovascular protection [20,21]. Moreover, it must be noted that the omega-6/omega-3 fatty acids ratios fall within the recommended values (<10) [22], and those of the *T. fragrantissimus* and *T. pulegioides* species (0.54 and 0.75, respectively) assume relevance due to its low value, which is believed to be associated with the prevention of many chronic diseases, including inflammatory bowel disease, rheumatoid arthritis, asthma, kidney disease, and several other inflammatory conditions [21,23].

Regardless of this generic similarity, specific features could be found among the plants. Linoleic acid was the most abundant fatty acid in *T. zygis*, while α-linolenic acid prevailed in the other species, with percentages of 28% and 36% in *T. pulegioides* and in *T. fragrantissimus*, respectively. Fernandes et al. [18] have found similar results for *T. pulegioides* (37% of α-linolenic acid) in a wild *T. pulegioides* plant. Nevertheless, the present data point to a lower prevalence of SFA and higher amounts of MUFA compared to that study. Naturally, differences may be related to multiple factors, including growth conditions (organic farming versus wild).

Table 1. Nutritional value of the three *Thymus* plant species.

Total Content	*T. zygis*	*T. pulegioides*	*T. fragrantissimus*
Total Carbohydrates (g/100 g dw)	86.88 ± 0.51	81.22 ± 0.90	77.56 ± 0.16
Protein (g/100 g dw)	3.59 ± 0.13	8.28 ± 0.81	10.49 ± 0.13
Ash (g/100 g dw)	5.02 ± 0.25	8.24 ± 0.06	7.66 ± 0.22
Minerals (mg/100 g dw) *			
Na	7.6	7.4	9.0
K	1229.3	2185.6	1719.5
Ca	566.0	1039.4	865.6
Mg	119.8	148.3	241.8
Fe	4.4	1.9	7.8
Mn	9.8	15.7	6.9
Cu	0.1	0.1	0.3
Zn	2.1	1.7	2.7
Fat (g/100 g dw)	4.52 ± 0.12	2.27 ± 0.03	4.30 ±0.19
Fatty acids (relative %) *			
C12: 0	0.48	0.5	0.7
C14: 0	1.2	1.1	1.4
C16: 0	18.8	23.6	23.3
C18: 0	3.2	3.3	2.8
C18: 1n9	16.9	19.0	8.6
C18: 2n6c	32.6	20.4	19.3
C18: 3n3	15.6	27.6	36.2
C20: 0	2.8	1.8	2.6
C22: 1n9	8.3	2.7	5.0
SFA	26.5	30.2	30.9
MUFA	25.3	20.9	13.8
PUFA	48.3	48.9	55.3
PUFA/SFA	1.8	1.6	1.8
n-6/*n*-3	2.1	0.8	0.5
Caloric content (kcal/100 g dw)	402.50 ± 0.42	378.39 ± 0.08	390.86 ± 1.83

Lauric acid (C12: 0); Myristic acid (C14: 0); Palmitic acid (C16: 0); Stearic acid (C18: 0); Oleic acid (C18: 1n9c + t); Linoleic acid (C18: 2n6c); α-Linolenic acid (C18: 3n3); Arachidic acid (C20: 0); Erucic acid (C22: 1n9); * S.E.M. < 10%.

2.2. Phenolic Characterization of *Thymus* Decoctions

The yield range of the decoctions extracts of the three *Thymus* species was between 12% and 25%, with minimum and maximum values corresponding to *T. zygis* and *T. pulegioides*, respectively (Table 2).

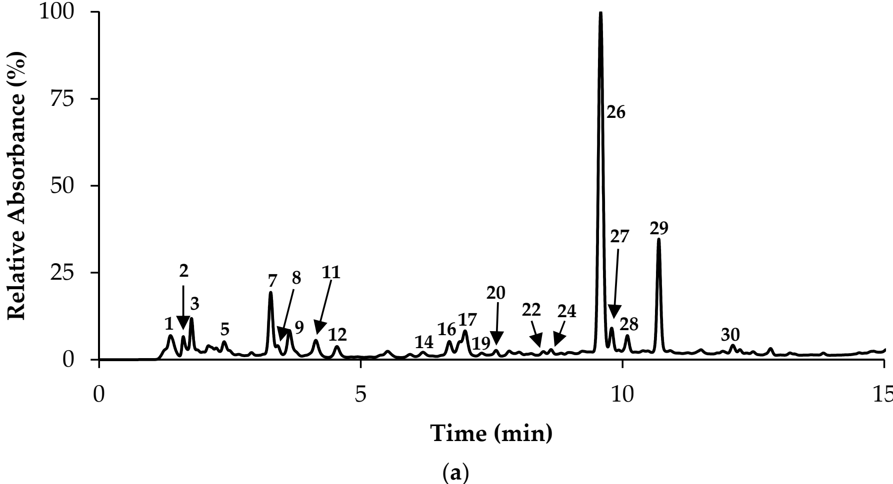
The decoction of *T. pulegioides* also showed a superior content of total phenolic compounds when compared to the others (391 versus 287–288 µg GAE/ mg extract, p-values < 0.01) or to decoctions of *Thymus herba-barona*, *Thymus pseudolanuginosus*, and *Thymus caespititius* (236–293 µg GAE/mg of extract) [24], or even to aqueous extracts obtained from *Thymus serpyllum* at 50 and 100 °C (79 and 91 µg GAE/mg extract, respectively) [25]. Moreover, the extraction yield of this decoction was close to the one reported by other authors for methanol or 70% ethanol extracts of *T. pulegioides* wild plants (24.6 and 22.5, respectively) and its phenolic richness was superior to the latter (391 versus 210 µg GAE/mg extract) [18,19].

Table 2. Yield and total phenolic compounds of aqueous extracts of *T. zygis*, *T. pulegioides* and *T. fragrantissimus*.

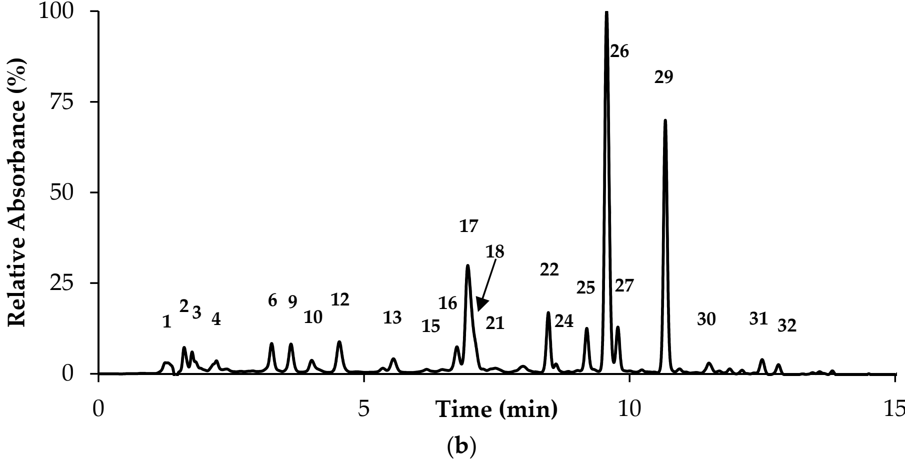
	T. zygis	*T. pulegioides*	*T. fragrantissimus*
Yield of Extraction (%)	12.39 ± 0.60 [a]	24.86 ± 1.71 [b]	15.67 ± 4.56 [a]
Total phenolic compounds (µg GAE/mg of extract)	287.86 ± 18.50 [a]	390.94 ± 2.48 [b]	287.08 ± 3.76 [a]

Mean values ± S.D.; Statistical analysis was performed by one-way ANOVA, followed by Tukey's test. In each line different letters ([a,b]) mean significant differences ($p < 0.05$).

The phenolic profiles of *T. zygis*, *T. pulegioides* and *T. fragrantissimus* decoctions were evaluated using UHPLC-ESI-DAD-MS^2/MS (Figure 2, Table 3). Moreover, the extracted ion chromatogram (EIC) and MS/MS spectrum of the main identified compounds are shown in Figure S2. Consistent with previous studies on *Thymus* polar extracts [3,24], these were mainly rich in rosmarinic acid (peak 26, UVmax at 290 and 328 nm, and $[M-H]^-$ at m/z 539). Interestingly, its levels (62–82 µg/mg extract, Table 3) were higher than those previously found for decoctions of *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* (40–56 µg/mg extract) [24], as well as for hydroethanolic extracts of *Thymus x citriodorus* (10 µg/mg extract) [26] and methanolic extracts of *Thymus praecox* (15 mg/g dry weight) [27]. The same tendency was found for caffeoyl rosmarinic acid isomers (MW 538, eluted in peaks 27, 29, 30, 31 and 32), which amounted from 16 to 63 µg/mg extract in these species, whereas they were only vestigial in *T. pseudolanuginosus* and *T. caespititius* or up to 15 µg/mg extract, in *T. herba-barona* decoctions [24]. Apart from that, levels of caffeoyl rosmarinic acid in hydroethanolic extracts of *T. x citriodorus* were reported to only reach about 2 µg/mg extract [26]. Notably, caffeic acid and its derivatives (rosmarinic acid, caffeoyl rosmarinic acid, salvianolic acid I/H) represented most of the phenolic components in *Thymus* aqueous extracts (p-values < 0.01), although they were less representative in *T. pulegioides* (64% of total phenolic compounds) when compared to the remaining species (close to 80%).



(**a**)



(**b**)

Figure 2. *Cont.*

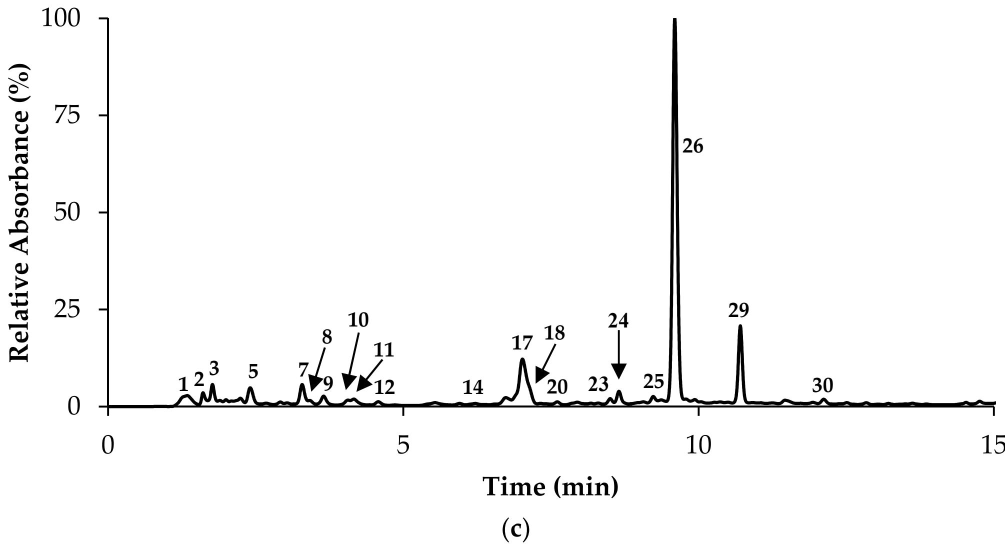


Figure 2. Chromatographic representation of *T. zygis* (**a**), *T. pulegioides* (**b**), and *T. fragrantissimus* (**c**) aqueous extracts at 280 nm. Numbers in the figure correspond to the UHPLC-DAD-ESI-MS^n peaks described in Table 3.

In turn, the *T. pulegioides* decoction was the richest in flavones (55.62 ± 1.05 µg/mg extract, *p*-values < 0.001), represented mainly by luteolin-*O*-glucuronide (peaks 17 and 21, $[M-H]^-$ at *m/z* 461→285), luteolin-*C*-glucoside (peak 12, MW 448), chrysoeriol-*O*-hexoside (peak 22, $[M-H]^-$ at *m/z* 461→299, 285) and apigenin-*O*-glucuronide (peak 25, MW 446, UVmax at 267 and 334). Overall, levels of flavones in *T. pulegioides* decoction were higher than those previously found in *T. herba-barona* and *T. caespititius* (22 and 32 µg/mg extract), but lower than *T. pseudolanuginosus* (73 µg/mg extract) [24].

The extract of this species was also noticeable due to its greater amounts of flavanones (23.68 ± 0.57 µg/mg extract, with statistically significant differences, *p*-values < 0.001), mainly comprised by eriodictyol-*O*-hexoside (factions 6 and 9, UVmax 283 nm, $[M-H]^-$ at *m/z* 449→287), naringenin-*O*-glucoside (peak 15, UVmax 283 nm, MW 434 and $[M-H]^-$ at *m/z* 433→271), that overall accounted for 19.93 ± 0.46 µg/mg extract. Please note that the compounds described herein (caffeic acid derivatives and flavonoids, including glycosidic forms) were previously detected in aqueous or methanolic/ethanolic extracts of other *Thymus* species [3,26].

Table 3. Identification and quantification of the compounds in the aqueous extracts of *T. zygis*, *T. pulegioides* and *T. fragrantissimus* by UHPLC-DAD-ESI-MS^n.

NP	RT (min)	λmax	Compound	$[M-H]^-$	MS^2 Main Fragments ESI-MS^n (*m*/*z*)	*T. zig*	*T. pul*	*T. fragr*
1	1.3	298	Quinic Ac	191	111, 173, 93, 85	D	D	D
2	1.6	277	Syringic Ac hex	359	197, 179, 135	D	D	D
3	1.8	281	Danshensu	197	179, 73	D	D	D
4	1.9	283	Eriod di-*O*-hex	611	449, 287	-	D	-
5	2.4	271, 326	Api di-*C*-glc	593	473, 503, 575, 353	1.60 ± 0.32 ^{a}	-	2.69 ± 0.50 ^{a}
6	3.3	283	Eriod-*O*-hex (isom1)	449	287	-	9.43 ± 0.21	-
7		289, 321	CaffAc	179	135	8.46 ± 0.02 ^{a}		2.54 ± 0.14 ^{b}
8	3.4	285	SA F der	375	313, 179	D	-	D
9	3.7	283	Eriod-*O*-hex (isom2)	449	287	6.02 ± 0.94 ^{a}	9.90 ± 0.19 ^{b}	2.71 ± 0.03 ^{c}
10	4.1	281, 342	Querc glcA	477	301	-	3.28 ± 0.03 ^{a}	1.13 ± 0.25 ^{b}
11	4.2	282, 342	Querc-*O*-hex	463	301	2.97 ± 0.90 ^{a}	-	1.48 ± 0.02 ^{b}
12	4.6	341	Lut-*C*-glc	447	357, 285, 327	4.86 ± 0.03 ^{a}	8.27 ± 0.13 ^{b}	2.00 ± 0.02 ^{c}
13	5.6	281, 342	SA I/H	537	339, 493	-	D	-
14	6.2	258, 268, 342	Lut-*O*-di glc	593	285, 447	1.19 ± 0.01 ^{a}	-	0.36 ± 0.02 ^{b}
15	6.2	283	Nar-*O*-glc	433	271, 313	-	1.90 ± 0.97	-
16	6.8	282, 333	Scut-*O*-glcA	461	285, 175, 284, 257	D	D	-
17	7.0	254, 265, 345	Lut-*O*-glcA (isom1)	461	285	7.57 ± 0.05 ^{a}	26.14 ± 0.78 ^{b}	16.86 ± 0.21 ^{c}
18	7.1	283	Eriod-*O*-glcA	463	287, 175, 151	-	D	D
19	7.3	282, 336	Querc-*O*-pent	433	301	D	-	-
20	7.6	288, 321	RA hex	521	359	D	-	D
21		254, 267, 344	Lut-*O*-glcA (isom2)	461	285	-	D	-
22	8.5	240, 339	Chrys-*O*-hex	461	299, 285	0.78 ± 0.01 ^{a}	12.00 ± 0.15 ^{b}	
23		281	CaffAc der	553	465, 311, 535, 357	-	-	D
24	8.7	283	Sagerinic Ac	719	359, 539, 521, 341	D	D	D
25	9.3	267, 334	Api-*O*-glcA	445	269, 175	D	9.20 ± 0.21 ^{a}	1.76 ± 0.10 ^{b}
26	9.6	290, 328	RA	359	223, 197, 179	62.36 ± 2.72 ^{a}	81.65 ± 7.02 ^{b}	81.04 ± 7.93 ^{b}
27	9.8	287, 311	CaffRA (isom1)	537	493, 515, 357, 335, 519, 153	2.79 ± 0.24 ^{a}	5.25 ± 0.19 ^{b}	-
28	10.1	287, 328	SA B	717	519, 357, 555, 359	1.89 ± 0.20	-	-
29	10.7	290, 323	CaffRA (isom2)	537	493, 359, 519, 179	19.40 ± 0.76 ^{a}	57.73 ± 1.95 ^{b}	16.21 ± 0.54 ^{c}
30	12.1	288, 322	CaffRA (isom3)	537	375, 493, 357, 359	D	D	D
31	12.5	287, 328	CaffRA (isom4)	537	439, 519, 357, 197, 493, 323, 331, 313	-	D	-
32	12.8	288, 323	CaffRA (isom5)	537	519, 359, 357, 339, 235, 493, 279, 207	-	D	-
					Total	119.90 ± 3.31 ^{a}	225.79 ± 15.08 ^{b}	126.65 ± 9.73 ^{a}
					Caffeic acid and derivatives	94.89 ± 1.84 ^{a}	144.63 ± 8.79 ^{b}	101.12 ± 6.75 ^{a}
					Flavones	16.01 ± 0.27 ^{a}	55.62 ± 1.05 ^{b}	21.67 ± 0.59 ^{c}
					Flavonols	2.97 ± 0.90 ^{a}	3.28 ± 0.03 ^{a}	2.62 ± 0.25 ^{a}
					Flavanones	6.02 ± 0.94 ^{a}	19.93 ± 0.46 ^{b}	2.71 ± 0.03 ^{c}

NF-Number of peak represented in Figure 2; D-Detected; *T. zig*-*T. zygis*; *T. pul*-*T. pulegioides*; *T. fragr*-*T. fragrantissimus*; Ac-acid; Api-Apigenin; CaffAc-Caffeic acid; Caff-Caffeoyl; Chrys-Chrysoeriol; Der-Derivative; Eriod-Eriodictyol; Glc-Glucoside; GlcA-Glucuronide; Hex-Hexoside; Lut-Luteolin; Nar-Naringenin; Pent-Pentoside; Querc-Quercetin; RA-Rosmarinic acid; SA-Salvianolic acid; Scut-Scutellarein; Values are expressed as μg/mg extract; In each line different letters (^{a,b,c}) mean significant differences ($p < 0.05$).

2.3. Antioxidant Activity

It is widely known that the antioxidant capacity of plant extracts are closely associated with phenolic components, which might interact with free radicals through electron or hydrogen donation [28]. In this study, the antioxidant potency of *Thymus* decoctions was screened by two generalized assays, namely the DPPH$^{\bullet}$ scavenging method and reducing power, which respectively evaluate the ability to trap the synthetic free radicals DPPH$^{\bullet}$ and to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). In general, the extracts presented a high antioxidant capacity, since EC_{50} values were up to twice as high as the commercial reference compounds (Table 4). Among the three extracts, *T. pulegioides* had the lowest DPPH$^{\bullet}$ EC_{50} value (9.50 ± 1.98 µg/mL), which corresponded to about 1.4-fold of ascorbic acid (EC_{50} = 6.9 ± 0.5), while *T. fragantissimus* and *T. zygis* were established at 13 µg/mL. This fact shows the greater ability of *T. pulegioides* to scavenge the free radical DPPH$^{\bullet}$ in comparison to the other two plant species and can possibly be associated with its higher phenolic contents. Interestingly, regardless of the higher ferric ion reduction tendency (EC_{50} = 30.73 ± 1.48 µg/mL), the superiority of this extract was not so clear, suggesting that non-phenolic components might also play a relevant role in this reaction.

Table 4. Antioxidant and antibacterial properties of aqueous extracts from *T. zygis*, *T. pulegioides* and *T. fragrantissimus*.

	T. zygis	*T. pulegioides*	*T. fragrantissimus*	Standard
Antioxidant properties				
DPPH$^{\bullet}$ [(1)] (µg/mL)	12.65 ± 2.30 ^{a,b}	9.50 ± 1.98 ^{a,b}	12.87 ± 3.79 ^{a}	6.90 ± 0.5 ^{b}
Reducing Power [(2)] (µg/mL)	33.66 ± 1.93 ^{a}	30.73 ± 1.48 ^{a}	32.44 ± 4.27 ^{a}	16.30 ± 1.5 ^{b}
Antibacterial properties [(3)] (MIC/MBC, mg/mL)				
G (+) bacteria				
Staphylococcus aureus	1.13/1.13	5.75/5.75	3.75/3.75	0.25/0.25
Staphylococcus epidermidis	4.50/4.50	5.75/11.50	7.50/>7.50	<0.06/<0.06
G (−) bacteria				
Salmonella typhimurium	4.50/4.50	5.75/11.50	7.50/7.50	0.50/0.50
Escherichia coli	>4.5/>4.5	>11.50/>11.50	7.50/>7.50	0.50/0.50
Pseudomonas aeruginosa	4.5/>4.5	5.75/11.50	7.50/>7.50	0.50/1.0

[(1)] Amount of extract required to reduce 50% of the 60 µM radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$); Ascorbic acid was used as reference compound; [(2)] Amount of extract able to provide 0.5 of absorbance by reducing 3.5 µM Fe^{3+} to Fe^{2+}; 2,6-Di-*tert*-butyl-4-methylphenol was used as reference compound; Mean values ± S.D.; Statistical analysis was performed by one-way ANOVA, followed by Tukey's test. In each line different letters (^{a,b}) mean significant differences ($p < 0.05$); [(3)] Nisin was used as reference compound. Mean values; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

The high antioxidant ability herein described for *T. pulegioides* decoctions exceeded that previously described for the methanolic extract from a wild *T. pulegioides* plant (EC_{50} = 680 ± 30 and 490 ± 30 µg/mL DPPH$^{\bullet}$ and reducing power assays, respectively) [18]. More recently, Kindl et al. [19] found promising results for an hydroethanolic extract of wild Croatian *T. pulegioides* plants, reporting DPPH$^{\bullet}$ EC_{50} = 4.18 ± 0.02 µg/mL and reducing power EC_{50} = 11.39 ± 0.07 µg/mL, which corresponded to about 1.7–2.5 times those of the reference commercial compounds [19]. To our knowledge, there are no literature data regarding the antioxidant abilities of aqueous extracts from *T. zygis*, *T. pulegioides* or *T. fragantissimus* origin. Still, the high abilities reported herein for decoctions are in line with those previously described by us for *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespititius* aqueous extracts [24]. However, Baharfar et al. [29] also observed that a *Thymus kotschyanus* aqueous extract could request DPPH$^{\bullet}$ with half the potency of ascorbic acid.

2.4. Antibacterial Activity

In opposition to *Thymus* essential oils, the antibacterial effects of polar extracts from *Thymus* plants have been scarcely exploited. In the present study, we evaluated the inhibitory activities of *T. zygis*,

T. pulegioides or *T. fragantissimus* aqueous extracts against the Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis*, and the Gram-negative bacteria *Salmonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa*, through the broth microdilution assay.

Interestingly, *T. zygis* decoctions were the most active among the three plant samples. As the levels of phenolic compounds in this decoction were below those of *T. pulegioides*, the results suggest that phenolic components do not lead this bioactivity. *Staphylococcus aureus* was the most sensitive species to thyme decoctions, with its growth and viability being inhibited at 1.13, 3.75, and 5.75 mg/mL for *T. zygis*, *T. fragantissimus*, and *T. pulegioides*, respectively (Table 4). The results, combined with those previously reported for *T. herba-barona, T. pseudolanuginosus*, and *T. caespititius* allow us to conclude that, among the six aqueous extracts of thyme [24], *T. herba-barona* and *T. zygis* (MIC/MBC of 0.6 mg/mL and of 1.13, respectively) are the two most active towards *Staphylococcus aureus*. Overall, MIC and MBC for the six thyme species against this bacterium were established in the range of 0.6 to 5.75 mg/mL, which is in accordance with the reported data for thyme extracts of distinct origins. In this context, Benbelaïd et al. [30] reported an MIC value of 1.0 mg/mL for an aqueous extract of *Thymus lanceolatus*, while MIC values of 6.25 mg/mL, 5.0 mg/mL, and 0.5 mg/mL were previously reported for ethanolic extracts of *Thymus vulgaris*, and methanolic and ethanolic extracts of *Thymus capitatus* [31–33].

Among the tested Gram-negative bacteria, *S. typhimurium* was the most susceptible to *Thymus* extracts. Both the growth and viability of *S. typhimurium* were inhibited at 4.5 mg/mL by *T. zygis* and at 7.5 mg/mL by *T. fragrantissimus*. Instead, *T. pulegioides* inhibited bacterial growth at 5.75 mg/mL, but its viability was only affected at higher levels (MBC 11.5 mg/mL). Combined with our previous results for *T. herba-barona*, *T. pseudolanuginosus*, and *T. caespetitius* decoctions (MIC in the range of 3.5–7 mg/mL) [24], it may be suggested that, to inhibit *S. typhimurium*, aqueous extracts of thyme should be used in high concentrations. These results are also consistent with those from other authors. In particular, Benbelaïd et al. [30] reported a MIC value of 4.0 mg/mL when *S. typhimurium* was treated with a water extract from *T. lanceolatus*. Values of the same magnitude were reported for a methanolic extract of *T. capitatus* (MIC = 6 mg/mL) [32], or hydroethanolic extracts from *T. vulgaris* and *Thymus caramanicus* (MIC values of 12.5 and 2.6 mg/mL, respectively) [31].

In turn, *P. aeruginosa* and *E. coli* were even more resistant to thyme extracts, albeit at different levels. While *P. aeruginosa* growth was inhibited by all samples (MIC values of 4.5, 5.75, and 7.5 mg/mL for *T. zygis*, *T. pulegioides*, and *T. fragantissimus*, respectively), only the latter species negatively impacted *E. coli* growth at 7.5 mg/mL. Moreover, *T. pulegioides* was able to kill *P. aeruginosa* (MBC = 11.5 mg/mL), while no effect on the viability of *E. coli* was observed. *Thymus* ethanolic extracts have been previously demonstrated to inhibit *P. aeruginosa*, with MIC values of 2.0 mg/mL (*T. capitatus*) [33] and 0.25 mg/mL (*T. lanceolatus*) [30], but no data on their ability to kill this bacterium were delivered. Likewise, ethanolic extracts of *T. capitatus*, *T. vulgaris*, *T. caramanicus*, and *T. lanceolatus* were shown to inhibit the growth of *E. coli* strains (MIC in the range of 0.5–8 mg/mL) [31,33] although no information was given about their effects on the viability of this bacterium.

3. Materials and Methods

3.1. Chemicals

Rosmarinic acid, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and eriodictyol-7-*O*-glucoside were obtained from Extrasynthese (Genay, France). Gallic acid, nisin, ascorbic acid and 2,6-Di-*tert*-butyl-4-methylphenol and the fatty acids methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; standard 47885-U) from Supelco (Bellefonte, PA, USA) were obtained from Sigma Chemical Co (St. Louis, MO, USA). Folin–Ciocalteu reagent, Na_2CO_3, formic acid and ethanol were purchased from Panreac (Barcelona, Spain). *n*-hexane, methanol, and acetonitrile with HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). Mueller–Hinton agar was obtained from VWR, Prolabo Chemicals (Radnor, PA, USA). Water was treated in a Direct-Q ® water purification system (Merck Life Science, Darmstadt, Germany).

3.2. Plant Cultivation and Material

The *T. zygis*, *T. fragantissimus*, and *T. pulegioides* species have been cultivated under an organic regime by Ervital (Mezio-Castro Daire, Portugal, GPS coordinates 40.976351, −7.903492). The cultivation soil was of granitic origin, medium to coarse in texture. It was moderately acidic (pH in water 5.6), with an organic matter content of 3.9%, with low levels of extractable phosphorus (18 ppm) and high levels of extractable potassium (110 ppm). During the time of cultivation (April 2014–June 2015), local temperatures ranged from 2 °C to 27 °C. Minimum values were registered in winter (November 2014 to February 2015, 2 °C–12 °C), while temperatures in the spring varied between 11 °C to 22 °C and reached a maximum of 27 °C in June 2015. Relative humidity varied from 2% to 90% with 29% on average, while the average total precipitation was 75 mm (65–94 mm).

No chemical or bacteriological treatment was applied to the plants. Their appearance and behavior were regularly monitored to ensure their health and the absence of pathogens. Whenever appropriate, weeds were removed from the surrounding soil.

The aerial parts of each individual *Thymus* (flowers, leaves, and stems) were collected and dried in a ventilated incubator at 20 to 35 °C for three to five days. After drying, the plants were transported in Kraft paper-type bags to the lab, where they were kept frozen until use.

3.3. Extraction of Phenolic Compounds

The extraction of phenolic compounds was performed according to the method described by Ferreira et al. [34], with adaptations. Briefly, 0.5 mm mesh powder of the aerial parts (flowers, leaves, and stems) of *T. zygis*, *T. fragantissimus*, and *T. pulegioides* (5 g) were extracted for 15 min using a decoction 20:1 (5 g in 100 mL of water), filtrated, and concentrated using a rotary evaporator. The concentrate was defatted with *n*-hexane (1:1 *v*/*v*) and freeze-dried. This procedure was performed in three independent assays.

3.4. Nutritional Value

The chemical composition of samples was determined according to AOAC methods [35]. Crude protein content (N × 6.25) of the samples was estimated through the macro-Kjeldahl method. Ash content was determined by incineration in a muffle furnace at 550 °C for 6 h and gravimetric quantification. Crude fat was obtained by Soxhlet extraction with light petroleum for 8 h, followed by filtration through a 0.2 µm nylon filter, solvent removal in a rotary evaporator, and drying, followed by gravimetric quantification. Total carbohydrates were calculated by difference. Calorific content was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat) [18].

For mineral determination, approximately 1 g of ash was digested with nitric acid. After digestion, samples were filtered and the volume adjusted to 100 mL with ultrapure water. Minerals (Na, K, Ca, Mg, Fe, Mn, Cu, and Zn) were quantified in a Perkin Elmer Analyst 100 flame atomic absorption spectrometer (Villepinte, France) equipped with a single hollow cathode lamps for each element and an air-acetylene burner [36].

Fatty acids were analyzed by transesterification of the fat fraction in the presence of sodium methoxide. The crude fat (1 g) was placed in a 15-mL glass tube and rod crushed and dissolved in 6 mL of *n*-heptane. Next, 400 µL of 0.2 M sodium methoxide were added to each sample and the tube was vigorously stirred in a vortex for 1–2 min at room temperature, allowing the formation of fatty acid methyl esters (FAME) from triglycerides. After decanting, sample supernatants were filtered through a 0.2 µm nylon filter from Milipore and injected in the gas chromatography system. Fatty acids were analyzed in a DANI 1000 gas chromatographer (GC) equipped with a split/splitless injector and a flame ionization detector (FID), following the method used by Fernandes et al. [18]. The identification was performed by comparing the relative retention times from samples with FAME peaks (standard mixtures). The results were recorded and expressed as a relative percentage of each fatty acid.

3.5. Identification and Quantification of Phenolic Compounds

The total phenolic content of each *Thymus* extract was determined according to the adapted Folin–Ciocalteu colorimetric method, as described by Pereira et al. [37]. The individual phenolic compounds were identified through UHPLC-DAD-ESI-MS^n analysis of extracts (5 mg/mL), as described by Afonso et al. [24]. Elution was carried out with a mixture of 0.1% (*v*/*v*) of formic acid in water (solvent A) and acetonitrile (solvent B) and the flow rate used was 0.2 mL/min^{-1}, following the same program. UV–Vis spectral data for all peaks accumulated in the range 200–600 nm and the chromatographic profiles were recorded at 280, 320, and 340 nm. The mass spectrometer used was a Thermo LTQ XL (Thermo Scientific, San Jose, CA, USA) ion trap MS, equipped with an ESI source with Thermo Xcalibur Qual Browser software. The instrument was operated in negative-ion mode and the full scan covered the mass range from m/z 100 to 2000. Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. CID–MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 25–35 arbitrary units.

For quantitative experiments, the limits of detection (LOD) and quantification (LOQ) were calculated from the parameters of the calibration curves obtained by injection of known concentrations of the exact or structurally related standard compounds represented in Table S1.

3.6. Antioxidant Activity

3.6.1. DPPH$^\bullet$Scavenging Test

The scavenging capacity of different concentrations (0.05–0.8 mg/mL) of *T. zygis*, *T. fragantissimus*, and *T. pulegioides* extracts was tested using a DPPH radical test, as described before [38]. Ascorbic acid was used as the positive control.

3.6.2. Reducing Power Test

The ability of *T. zygis*, *T. fragantissimus*, and *T. pulegioides* (0.05–0.25 mg/mL) aqueous extracts to reduce iron (III) was assessed by the method previously described [39]. 2,6-Di-*tert*-butyl-4-methylphenol was used as the positive control.

3.7. Antimicrobial Activity

The antibacterial potential of the *Thymus* polar extracts was evaluated against five bacterial strains, including Gram-positive bacteria (*S. epidermidis* NCTC 11047 and *S. aureus* NCTC 6571) and Gram-negative bacteria (*S. typhimurium* NCTC 12023, *E. coli* NCTC 9001, and *P. aeruginosa* NCTC 10662) from the National Collection of Type Cultures, operated by Public Health England. All strains were cultured in Mueller–Hinton agar and incubated at 37 °C for 24 h.

The MIC and MBC of aqueous solutions of *T. zygis*, *T. fragantissimus*, and *T. pulegioides* were determined by the broth microdilution method, using an adapted method previously described by Afonso et al. [24]. Nisin, an antibacterial polypeptide approved as a food preservative, was used as the positive control [40].

3.8. Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's test were used to detect any significant differences among different means. Alternatively, Student's *t* test was used to determine the significant difference between two different samples. A *p*-value under 0.05 was assumed to indicate a significant difference. The results were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

4. Conclusions

The nutritional composition, phenolic profile, antioxidant and antimicrobial activities of three species of *Thymus* grown under organic farming are reported within this study. Based on our results, it is possible to infer that these thyme species present a richness of polyunsaturated fatty acids and an interesting omega-6/omega-3 fatty acids ratio, along with high K/Na ratios that can contribute to cardiovascular protection. *T. pulegioides* presented the highest antioxidant capacity, consistent with its high content of polyphenols, which were mainly composed of rosmarinic acid and caffeoyl rosmarinic acid, also showing considerable levels of flavones (luteolin-*C*-glucoside, luteolin-*O*-glucuronide, and apigenin-*O*-glucuronide) and flavanones (eriodictyol-*O*-hexoside isomers). In addition, aqueous extracts obtained from these *Thymus* species, particularly *T. zygis*, showed antibacterial potential.

Supplementary Materials: Supplementary materials can be found in a separate file: Figure S1: Pictures of *Thymus zygis* (a), *Thymus pulegioides* (b) and *Thymus fragrantissimus* (c); Figure S2: Extracted ion chromatograms and (inset) mass spectrum of ESI–MS^2 of the corresponded ion of main phenolic compounds identified in *Thymus zygis*, *Thymus pulegioides* and *Thymus fragrantissimus* by UHPLC-DAD-ESI-MS^n; Table S1: Linearity, LOD and LOQ of the standard compounds used as references.

Author Contributions: A.F.A. and O.R.P. performed the majority of the experimental work, the analysis, and interpretation of data, and wrote the paper; M.V. contributed with mineral analysis; A.M.S.S. co-wrote the manuscript; S.M.C. coordinated the work and co-wrote the paper.

Acknowledgments: The authors wish to acknowledge the Foundation for Science and Technology (FCT), the European Union, the National Strategic Reference Framework (QREN), the European Regional Development Fund (FEDER), and Operational Programme Competitiveness Factors (COMPETE), for funding the Organic Chemistry Research Unit (QOPNA) (FCT UID/QUI/00062/2013) and Mountain Research Center (CIMO) (UID/AGR/00690/2013), through national funds and where applicable co-financed by FEDER, within the PT2020 Partnership Agreement. Susana Cardoso wishes to thank the research contract under the project AgroForWealth: Biorefining of agricultural and forest by-products and wastes: integrated strategic for valorisation of resources towards society wealth and sustainability (CENTRO-01-0145-FEDER-000001), funded by Centro2020, through FEDER and PT2020.

Conflicts of Interest: The authors declare no conflict of interest.

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Decocciones de *Salvia elegans, Salvia greggii* y *Salvia officinalis*: actividades antioxidantes e inhibición de enzimas metabólicas

Objetivos

El presente estudio tiene como objetivo dilucidar la composición fenólica y los efectos biológicos de las decocciones de *Salvia elegans* y *Salvia greggii*, centrándose principalmente en su potencial antioxidante y en las actividades en enzimas metabólicas con un impacto relevante en la obesidad y la diabetes, al mismo tiempo que se comparan con la especie bien conocida de *Salvia officinalis*.

1. Introducción

Los géneros de *Salvia* (*Salvia* spp.), pertenecientes a la familia Lamiaceae, comprenden más de 900 especies que se utilizan para distintos fines, incluida la industria culinaria, cosmética o la medicina tradicional debido a sus beneficios para la salud [1,2]. Entre ellos, *S. officinalis*, es decir, la salvia común o *Salvia* dálmata, se cultiva ampliamente, y ha sido la especie mejor investigada en lo que respecta al potencial biológico, así como de sus aceites esenciales y extractos polares. En estudios *in vivo* y *in vitro* con esta especie de *Salvia*, se obtuvieron resultados prometedores que se enfocaron en su efecto sobre la memoria y las funciones cognitivas, perfil bioquímico de glucosa y lípidos [3], actividad antiinflamatoria [4,5], actividad antioxidante [6,7], o la citotoxicidad frente a distintas células cancerígenas [8,9]. Sin embargo, muchas especies menos ampliamente distribuidas, como *S. greggii* A. Gray y *S. elegans* Vahl., siguen estando poco estudiadas. *S. greggii*, (conocida como "salvia de otoño") y *S. elegans* son especies originarias de México y actualmente se cultivan en los Estados Unidos y otras regiones del mundo [10,11]. Estas dos especies de plantas se usan ampliamente en la medicina tradicional, particularmente en forma de infusiones o decocciones, para tratar problemas digestivos y orales (*S. greggii*) [1] o para disminuir la presión arterial y combatir los trastornos del sistema nervioso central como la ansiedad y el insomnio (*S. elegans*) [12–14]. Sin embargo, hasta donde se sabe, la composición fitoquímica y los efectos beneficiosos de los extractos polares, en particular los relacionados con el uso tradicional (acuoso), permanecen sin explorar. Por lo que sabemos, *S. greggii* ha sido evaluada en busca de compuestos terpénicos [15,16], y los extractos polares de *S. elegans* han sido objeto de atención, y en particular, se han demostrado sus efectos antihipertensivos, antidepresivos y ansiolíticos [12–14].

2. Métodos

2.1. Plantas y extracción de compuestos fenólicos

Las partes aéreas de las plantas *S. elegans, S. greggii* y *S. officinalis* se cultivaron bajo un régimen orgánico y se secaron en una incubadora ventilada a 20-35ºC, durante 3 a 5 días. Los extractos fenólicos se han obtenido utilizando una decocción en la proporción 1:20. Seguidamente las soluciones se filtraron, se concentraron, se evaporaron, se congelaron a -20°C y se liofilizaron. Se obtuvieron tres extractos de cada planta.

2.2 Identificación y cuantificación de compuestos fenólicos

Los extractos fueron caracterizados y cuantificados por cromatografía líquida de alta eficiencia, asociada a espectrometría de masas por electrospray en el modo de ionización negativo (UHPLC-DAD-ESI / MS^n).

2.3 Pruebas de bioactividad

2.3.1 Capacidad antioxidante

La actividad antioxidante de los extractos acuosos de las tres salvias se evaluó mediante seis ensayos químicos [17,18]: i) eliminación de radicales libres estables como el 2,2-difenil-1-picrililhidracilo ($DPPH^{\bullet}$), anión superoxido ($O_2^{\bullet-}$) y óxido nítrico ($NO^{\bullet}$); ii) el poder reductor por la reducción de hierro (III); iii) la capacidad de absorción de radicales de oxígeno (ORAC), que evalúa la actividad antioxidante a través de la inhibición de la oxidación, inducida por el radical peroxil ($RO_2^{\bullet}$); y la inhibición de la actividad de la xantina oxidasa (XO) [19], es decir, la enzima que cataliza la oxidación de la hipoxantina a la xantina, y además cataliza la xantina al ácido úrico con una producción concomitante de $O_2^{\bullet-}$.
En las pruebas DPPH y $NO^{\bullet}$ se utilizó ácido ascórbico como control positivo; en los métodos del poder reductor, $O_2^{\bullet-}$ y de inhibición de xantina oxidasa (XO) se utilizaron hidroxianisol butilado (BHA), ácido gálico y alopurinol como estándares, respectivamente. Los resultados de la prueba ORAC se expresaron en µM de equivalentes de trolox (µM TE) por mg de extracto. Los restantes resultados se expresaron como valores de EC_{50} (concentración de la muestra que proporciona el 50% de la actividad antioxidante) en µg por ml de extracto.

2.3.2 Inhibición de las actividades enzimáticas

La actividad enzimática metabólica de los tres extractos acuosos de salvias se evaluaron *in vitro* mediante la inhibición de la actividad de tres enzimas, la α-glucosidasa, la α-amilasa y la lipasa pancreática [20,21]. En las pruebas de inhibición de las actividades α-glucosidasa y α-amilasa se utilizó acarbosa como control positivo, mientras que en la prueba de inhibición de la lipasa pancreática se utilizó orlistat como estándar. En el método de inhibición de la actividad de α-glucosidasa, los resultados se expresaron en valores de EC_{50} (µg/ml), que corresponden a la concentración de la muestra que

proporcionó 50% de inhibición de la enzima. En las pruebas de inhibición de α-amilasa y lipasa pancreática se determinó el porcentaje de inhibición de las enzimas en 0.5 y 0.2 mg/ml de cada muestra de *Salvia*, respectivamente, o el EC_{50} para los compuestos de referencia.

3. Resultados y discusión

3.1. Compuestos fenólicos

Los rendimientos de las tres decocciones de *Salvia* fueron aproximadamente del 20%, con niveles ligeramente más altos observados para *S. elegans* y *S. greggii* en comparación con *S. officinalis* (22.1% y 22.2% *vs.* 19.3%, respectivamente). Consistente con su prevalencia en varias plantas de *Salvia* [1,2,22,23], los derivados del ácido cafeico (particularmente el ácido rosmarínico) fueron compuestos dominantes en las decocciones de *S. officinalis, S. elegans* y *S. greggii*, que representan aproximadamente un tercio de las especies fenólicas identificadas (Tabla 1). Sin embargo, se encontraron diferencias significativas entre los extractos de las tres plantas. De hecho, *S. elegans* se distinguió por su riqueza en derivados del ácido cafeico, a saber, ácido rosmarínico, cafeoil ácido rosmarínico, ácido salvianólico B (fracciones 36, 39 y 27, respectivamente), que en general fueron alrededor de 70% del total de los fenólicos cuantificados.

Tabla 1. Identificación y cuantificación de *S. officinalis, S. elegans* y *S. greggii* por UHPLC-DAD-ESI-MS^n

Fracción	TR (min)	λmax (nm)	$[M-H]^-$	Compuesto	mg/g de extracto		
					S. off	***S. ele***	***S. gre***
1	1.5	275	149	2,4-Dimetil ácido benzóico	4.1 ± 0.2	5.1 ± 0.1	7.3 ± 0.2
2	1.7	205	191	Ácido quinico	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.01
3	3.6	280	197	Danshensu	D	D	D
4	5.0	290, 324	353	*cis* 3-*O*- Ácido cafeioilquinico	-	-	3.10 ± 0.03
5	5.7	220, 278	137	Ácido hidroxibenzoico	D	2.0 ± 0.1	-
6	8.3	313	295	*p*- ácido pentosido de coumaroil	-	-	0.30 ± 0.02
7	8.8	290, 325	353	*Trans*-5-*O*- Ácido cafeoilquinico	-	-	1.9 ± 0.1
8	9.4	313	265	Derivado del ácido cumárico	-	-	2.90 ± 0.05
		ND	325	Cafeoil hexósido	D	-	-
9	9.7	290, 323	179	Ácido cafeico	1.80 ± 0.04	1.50 ± 0.02	-
10	9.8	314	325	Cumaroil hexósido	D	-	-
11	9.9	255, 265, 350	625	Quercetina di-hexósido	-	-	D
12	12.1	271, 336	593	Apigenina-6-*C*-glucósido-7-*O*-glucósido	4.3 ± 0.1	-	-
13	13.1	291, 311	637	Derivado del ácido ferúlico	-	D	-
14	13.5	274	571	Ácido yunnanico E (isómero 1)	-	1.9 ± 0.1	-
15	13.9	256, 267, 345	447	Luteolina-*C*-hexósido	-	-	4.60 ± 0.09

16	13.9	281, 345	477	Hidroxi-luteolina-glucurónido	D	1.9 ± 0.2	-
17	14.1	276	571	Ácido yunnanico E (isómero 2)	D	-	-
18	14.4	269, 304	473	Ácido cichorico	-	1.60 ± 0.07	-
19	14.8	267, 345	621	Apigenina-di-glucurónido	4.6 ± 0.3	-	-
20	15.2	268, 336	431	Apigenina-*C*- hexósido	-	-	15.7 ± 0.3
21	15.4	274	555	Ácido salvianólico K	1.6 ± 0.2	-	-
			571	Ácido yunnanico E (isómero 3)	D	-	-
22	15.8	255, 350	463	Quercetina-*O*-hexósido	-	-	2.7 ± 0.2
23	15.9	280, 333	461	Scutelareina-*O*-glucurónido	13.4 ± 0.6	3.9 ± 0.1	-
24	16.0	255, 265, 348	447	Luteolina-7-*O*-glucósido	-	-	26.1 ± 0.9
25	16.1	255, 266, 345	461	Luteolina-7-*O*-glucurónido (isómero 1)	8.4 ± 0.3	5.1 ± 0.3	-
26	16.9	271, 306	521	Salviaflasida	-	D	-
27	17.2	278	717	Ácido salvianólico B (isómero 1)	-	7.8 ± 0.4	-
28	17.3	279	571	Ácido yunnanico E (isómero 4)	0.9 ± 0.1	-	-
29	17.7	279	717	Ácido salvianólico (isómero 2)	-	1.7 ± 0.6	-
30	17.9	268, 334	577	Apigenina-rutinósido	4.5 ± 0.1	D	-
		283	719	Ácido sagerinico	6.0 ± 0.3	D	-
31	18.1	271, 304	717	Ácido salvianólico B (isómero 3)	-	1.7 ± 0.1	-
32	18.1	269, 329	431	Apigenina-hexósido	D	-	3.4 ± 0.2
33	18.4	267, 337	445	Apigenina-glucurónido	48.4 ± 1.3	3.2 ± 0.5	-
34	18.6	254, 266, 345	533	Luteolina malonil hexósido	-	-	D
35	18.6	270, 291, 326	717	Ácido salvianólico B (isómero 4)	D	-	-
36	19.0	218, 290, 328	359	Ácido rosmarínico	28.3 ± 0.6	35.5 ± 0.8	10.9 ± 0.2
37	19.2	269, 307, 343	461	Luteolina-*O*-glucurónido (isómero 2)	-	1.8 ± 0.1	
38	20.8	293, 328	373	Rosmarinato de metilo	-	-	D
39	21.2	290, 333	537	Cafeoil ácido rosmarínico/ Ácido salvianólico I (isómero 1)	1.2 ± 0.1	17.9 ± 0.1	-
40	21.4	293, 328	329	Derivado del ácido cafeico	-	-	5.0 ± 0.03
		239, 285, 330	537	Cafeoil ácido rosmarínico (isómero 2)	-	1.3 ± 0.04	-
41	22.1	295, 325	713	Derivado del ácido cafeico	-	D	-
42	22.3	280	537	Cafeoil ácido rosmarínico (isómero 3)	-	0.7 ± 0.04	-
43	23.0	289, 327	717	Ácido salvianólico B isómero	D	2.4 ± 0.04	-
				Ácido cafeico *y derivados*	39.8 ± 0.9	74.1 ± 0.5	20.8 ± 0.3
				Ácido cumárico *y derivados*	-	-	3.20 ± 0.06
				Flavonas	83.5 ± 2.3	15.9 ± 0.9	49.7 ± 1.3
				Flavonoles	-	-	2.7 ± 0.2

D: Detectado; TR: tiempo de retención; *S. off—S. officinalis*; *S. ele—S. elegans*; *S. gre—S. greggii.*

El extracto acuoso de *S. greggii* se caracterizó por altos porcentajes de flavonas glicosídicas, en particular luteolina-*O*-hexósido y apigenina-*C*-hexósido (fracciones 24 y 20, respectivamente); y de cantidades moderadas de luteolina-*C*-hexósido, flavonol quercetina-*O*-hexósido y de dos derivados de ácido cumárico (fracciones 15, 22, 6 y 8, respectivamente). Ninguno de estos compuestos se detectó en las decocciones de las otras dos especies. Por lo tanto, a nivel general los extractos obtenidos de *S. elegans* y *S. greggii* fueron claramente distintos de los de *S. officinalis*, con esta última dominada por la forma *O*-hexurónica de apigenina y scutelareina, junto con ácido rosmarínico (48.4, 13.4 y 28.3 mg/g de extracto, respectivamente). Es de señalar que el predominio de apigenina-*O*-glucurónido y ácido rosmarínico en esta decocción es coherente con la abundancia de estos dos constituyentes previamente descritos en extractos polares de esta especie [2,24], sin embargo, es curioso que esta sea la primera vez que se detecte scutelareina-*O*-glucurónido en extractos de *S. officinalis*, mientras que los estudios anteriores solo describieron su forma de aglicona.

3.2. Actividad antioxidante

En general, la decocción de *S. elegans* fue más prometedora que la de *S. greggii* en lo que se refiere a su capacidad para eliminar los radicales libres y reducir el Fe^{3+} (Tabla 2). *S. elegans* presentó valores de EC_{50} aproximadamente 1.8–2.5 más bajos que *S. greggii* en $DPPH^{\bullet}$, $NO^{\bullet}$, $O_2^{\bullet}$ y en la prueba de poder reductor, y una tendencia a una mayor habilidad para capturar radicales peroxil (RO_2).

Tabla 2. Propiedades antioxidantes de las plantas *S. officinalis, S. elegans*, and *S. greggii*

	S. officinalis	***S. elegans***	***S. greggii***	**Standard**
$DPPH^{\bullet}$ (EC_{50} µg/mL) [(1)]	34.8 ± 3.3^{a}	10.7 ± 2.1^{b}	21.1 ± 2.5^{c}	6.69 ± 0.7^{b}
Reducing Power (EC_{50} µg/mL) [(2)]	40.0 ± 11.2^{a}	$31.3 \pm 5.0^{a,c}$	77.9 ± 5.6^{b}	16.30 ± 1.5^{c}
$NO^{\bullet}$ (EC_{50} µg/mL) [(1)]	118.2 ± 16.4^{a}	91.5 ± 14.5^{a}	167.8 ± 23.9^{b}	212.1 ± 9.7^{c}
$O_2^{\bullet -}$ (EC_{50} µg/mL) [(3)]	32.8 ± 0.6^{a}	30.6 ± 1.3^{a}	61.7 ± 3.4^{b}	7.8 ± 0.5^{c}
ORAC (µM TE/mg ext) [(4)]	404.4 ± 1.80^{a}	373.1 ± 28.1^{a}	335.6 ± 69.6^{a}	-
Xanthine oxidase (EC_{50} µg/mL) [(5)]	55.1 ± 10.6^{a}	71.8 ± 3.8^{b}	$70.1 \pm 4.0^{a,b}$	0.09 ± 0.01^{c}

[(1)] Se utilizó ácido ascórbico como control positivo. [(2)] Cantidad de extracto capaz de proporcionar 0.5 de absorbancia reduciendo 3.5 µM Fe^{3+} a Fe^{2+}. Se utilizó BHA como control positivo. [(3)] Se utilizó ácido gálico como como compuesto de referencia. [(4)] TE— equivalentes de trolox. [(5)] Se utilizó alopurinol como compuesto de referencia. Valores medios ± SD; El análisis estadístico se realizó mediante ANOVA de una vía, seguido de la prueba de Tukey. En cada fila, letras diferentes significan diferencias estadísticas significativas ($p < 0.05$).

El extracto de *S. elegans* también presentó un potencial antioxidante mejor que *S. officinalis*, con valores EC_{50} que presentaban una tendencia decreciente en las pruebas de $NO^{\bullet}$, $O_2^{\bullet}$ y poder reductor, y incluso tres veces más bajo para el ensayo DPPH. Además, hay que destacar que la potencia de *S. elegans* para contrarrestar el $DPPH^{\bullet}$ y

el $NO^•$ fue mayor que la del ácido ascórbico. La única excepción se observó para el ensayo ORAC, para el cual el resultado observado para *S. officinalis* fue mejor que el de *S. elegans*, aunque no fue estadísticamente significativo.

La Tabla 3 resume los coeficientes de correlación entre las cantidades de clases de componentes fenólicos encontrados en las decocciones de *Salvia* y los datos de los distintos experimentos biológicos. De acuerdo con estos resultados, es posible sugerir que la actividad antioxidante superior de la decocción de *S. elegans* está fuertemente asociada con su riqueza en ácido cafeico y derivados, ya que los factores de correlación en los ensayos $DPPH^•$, poder reductor, $NO^•$ y $O_2^{•-}$ fueron 0.801, 0.948, 0.986 y 0.844, respectivamente.

Tabla 3. Coeficientes de correlación entre las cantidades de componentes fenólicos encontrados en las decocciones de *Salvia* (ácido cafeico y derivados, derivados de ácido cumárico, flavonas y flavonoles) y los datos de los distintos experimentos biológicos.

	DPPH	RP	ORAC	NO	O2	XO	AG	L
Flavones	−0.971	−0.357	0.454	−0.498	−0.123	0.901	−0.551	−0.367
Flavonols	−0.239	−0.934	−0.891	−0.868	−0.992	−0.434	−0.835	0.930
CafAcD	0.801	0.948	0.400	0.986	0.844	−0.237	0.995	−0.485
CouAcD	−0.239	−0.934	−0.891	−0.868	−0.992	−0.434	−0.835	0.930
DPPH		0.570	−0.228	0.690	0.356	−0.771	0.734	0.134
RP			0.670	0.988	0.971	0.084	0.976	−0.738
ORAC				0.547	0.829	0.796	0.493	−0.995
NO					0.922	−0.071	0.998 *	−0.624
O2						0.321	0.996	−0.878
XO							−0.134	−0.735
AG								−0.574

Valores de coeficiente de correlación de Pearson R; AG: actividad inhibidora de la α-glicosidasa; CafAcD — ácido cafeico y derivados; CouAcD: derivados del ácido cumárico; DPPH - actividad de captación de radicales DPPH; L - actividad inhibidora de la lipasa; NO — capacidad de eliminación de óxido nítrico; ORAC: capacidad de absorción de radicales de oxígeno; O2: actividad de captación de aniones superóxido; RP: poder reductor; XO: actividad inhibidora de la xantina oxidasa; * $p < 0.05$.

Los extractos polares de *S. officinalis* se han utilizado comúnmente como una referencia para la evaluación de las propiedades antioxidantes de otras plantas menos investigadas [25,26]. Por lo tanto, se podría concluir que, de acuerdo con otros estudios para extractos polares de varias especies de *Salvia*, las decocciones de *S. officinalis, S. elegans* y *S. greggii*, tienen una alta capacidad para eliminar el $DPPH^•$, con *S. elegans* mostrando la actividad más prometedora, seguido por *S. greggii* y *S. officinalis*. Aunque la prueba DPPH sea la más frecuentemente estudiada [25–28], los extractos polares obtenidos de las plantas de *Salvia* también se han analizado previamente para

determinar su capacidad antioxidante a través de varios otros ensayos, como el poder reductor [29], y la eliminación de radicales libres $NO^{•}$ [30] y $O_2^{•-}$ [31].

Del mismo modo, las decocciones de S*. officinalis, S. elegans* y *S. greggii* mostraron una gran capacidad para eliminar $RO_2^{•}$ (335–404 µM TE/mg) y para inhibir la actividad de la XO, aunque con menor potencia que el medicamento comercial alopurinol. Entre los extractos, *S. officinalis* fue la mejor, un hecho que podría estar relacionado con su riqueza en glucurónido de apigenina o en otras flavonas (es decir, glicósidos de escutellareina y luteolina), ya que estos compuestos se han descrito como inhibidores fuertes de esta enzima [32,33]. Aunque el efecto individual de los compuestos no ha sido probado por nosotros, los coeficientes de correlación entre los ensayos de antioxidantes y los compuestos principales de cada extracto acuoso están en buena concordancia (Tabla 3). En el ensayo inhibidor de XO, el contenido de flavonas de la decocción de *S. officinalis* presentaba una correlación significativa (0.901) con su capacidad para inhibir esta actividad enzimática.

3.3. Actividad enzimática metabólica

Las α-glucosidasa, α-amilasa y lipasa pancreática son enzimas digestivas involucradas en el metabolismo de los carbohidratos y los lípidos, que se asumen como puntos de control terapéutico en la obesidad y la diabetes. α-Amilasa y α-glucosidasa catalizan la hidrólisis de los carbohidratos en azúcares simples, por lo que su inhibición retrasa la digestión del almidón y los oligosacáridos contribuyendo a la reducción del aumento posprandial en los niveles de glucosa en plasma. A su vez, la inhibición de la lipasa disminuye la digestión de los triglicéridos de la dieta, lo que reduce los niveles de ácidos grasos libres y monoacilgliceroles en el intestino [34–36].

Tabla 4. Propiedades inhibitorias enzimáticas de decociones de las plantas *S. officinalis, S. elegans*, and *S. greggii*

	S. officinalis	*S. elegans*	*S. greggii*	Standard
α-Glucosidasa (EC_{50} µg/mL) (1)	71.2 ± 5.0^a	36.0 ± 2.7^b	345.3 ± 6.4^c	357.8 ± 12.3^c
α-Amilasa (2)	-	-	6.5 ± 3.0	0.7 ± 0.2
Lipasa pancreática (3)	4.6 ± 3.6^a	8.2 ± 0.3^a	14.4 ± 7.4^a	1.8 ± 0.4

(1) Acarbose se utilizó como estándar (2) Los resultados se expresan como porcentaje (%) de inhibición a la concentración de 0,5 mg/ml (decocciones de *Salvia*) o como EC_{50} (µg/ml), para el compuesto de referencia acarbosa. (3) Los resultados se expresan como porcentaje (%) de inhibición a la concentración de 0.2 mg/mL (decocciones de *Salvia*) o como EC_{50} (µg/ml), para el compuesto de referencia En cada línea, letras diferentes significan diferencias significativas ($p < 0.05$).

En este estudio, se evaluó la capacidad de las decocciones de *S. officinalis, S. elegans* y *S. greggii* para inhibir la actividad de estas tres enzimas digestivas. En particular, las

actividades inhibitorias de los tres extractos de *Salvia* contra la α-glucosidasa fueron muy prometedoras para *S. elegans* y *S. officinalis* (EC_{50} = 36.0 µg/mL y 71.2 µg/mL, respectivamente), demostrando actividades de 9 y 4 veces superiores que la droga antidiabética, acarbosa, respectivamente (Tabla 4).
Además, a pesar de menos eficaz que *S. elegans* y *S. officinalis*, la decocción de *S. greggii* fue tan efectiva como la acarbosa. Por lo tanto, nuestros resultados sugieren que las decocciones de *S. elegans, S. officinalis* y *S. greggii* podrían servir como agentes antidiabéticos y antiobesidad naturales para ayudar a controlar los niveles de glucosa a través del control de la actividad de la α-glucosidasa. Esto es consistente con estudios previos que informaron resultados idénticos para extractos polares de *Salvia* contra esta enzima [35,37].
Además, la capacidad inhibitoria de los extractos polares de las especies de *Salvia* hacia la α-glucosidasa se ha relacionado previamente con sus constituyentes fenólicos [38–40]. Tiendo en cuenta que el ácido rosmarínico y el caffeoil-ácido rosmarínico son dos componentes fenólicos principales en las decocciones de *S. elegans*, es factible suponer que podrían ser contribuyentes importantes para la mayor capacidad inhibitoria de este extracto. De hecho, se demostró una fuerte correlación entre los resultados obtenidos para las actividades inhibitorias de la α-glucosidasa y el contenido en ácido cafeico y derivados de los extractos (0.995). Curiosamente, también se observaron altos coeficientes de correlación entre la α-glucosidasa y los ensayos antioxidantes (Tabla 3), lo que sugiere que los efectos metabólicos y antioxidantes podrían posiblemente estar relacionados.
Sin embargo, a pesar de los datos de la literatura que parecen sugerir que algunos extractos polares de especies de *Salvia* pueden ser prometedores con respecto a sus habilidades para controlar la actividad de la α-amilasa [40] y la lipasa pancreática [41] [42], esto no fue lo observado para las decocciones de *S. officinalis*, *S. elegans* y *S. greggii* estudiadas en esta investigación.

4. Conclusiones

Este trabajo demostró que entre las tres plantas *S. elegans*, *S. greggii* y *S. officinalis*, las decocciones de *S. elegans* fueron las más prometedoras en cuanto a la actividad antioxidante y el potencial inhibitorio contra la α-glucosidasa, un hecho que podría estar relacionado con su riqueza en ácido cafeico y sus derivados. A su vez, se resalta la capacidad inhibitoria superior de *S. officinalis*, que posiblemente esté asociada con la presencia de flavonas. En conclusión, de manera similar que la bien conocida especie de *S. officinalis,* también *S. elegans* y *S. greggii* son una fuente valiosa de metabolitos

naturales y podrían usarse para aplicaciones comerciales en nuevos alimentos funcionales o ingredientes farmacéuticos dirigidos al tratamiento de la diabetes y la prevención de la obesidad.

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Article

Salvia elegans, *Salvia greggii* and *Salvia officinalis* Decoctions: Antioxidant Activities and Inhibition of Carbohydrate and Lipid Metabolic Enzymes

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Received: 30 October 2018; Accepted: 28 November 2018; Published: 1 December 2018

Abstract: *Salvia elegans* Vahl., *Salvia greggii* A. Gray, and *Salvia officinalis* L. decoctions were investigated for their health-benefit properties, in particular with respect to antioxidant activity and inhibitory ability towards key enzymes with impact in diabetes and obesity (α-glucosidase, α-amylase and pancreatic lipase). Additionally, the phenolic profiles of the three decoctions were determined and correlated with the beneficial properties. The *S. elegans* decoction was the most promising in regard to the antioxidant effects, namely in the scavenging capacity of the free radicals $DPPH^{\bullet}$, $NO^{\bullet}$ and $O_2^{\bullet-}$, and the ability to reduce Fe^{3+}, as well as the most effective inhibitor of α-glucosidase (EC_{50} = 36.0 ± 2.7 μg/mL vs. EC_{50} = 345.3 ± 6.4 μg/mL and 71.2 ± 5.0 μg/mL for *S. greggii* and *S. officinalis*, respectively). This superior activity of the *S. elegans* decoction over those of *S. greggii* and *S. officinalis* was, overall, highly correlated with its richness in caffeic acid and derivatives. In turn, the *S. officinalis* decoction exhibited good inhibitory capacity against xanthine oxidase activity, a fact that could be associated with its high content of flavones, in particular the glycosidic forms of apigenin, scutellarein and luteolin.

Keywords: sage; phenolic compounds; antioxidant; α-glucosidase; pancreatic lipase; α-amylase; LC-MS analysis

1. Introduction

Salvia genus (*Salvia* spp.), belonging to the Lamiaceae family, comprises more than 900 species that are used for distinct purposes, including the culinary and cosmetic industries or in traditional medicines due to their claimed health benefits [1,2]. Among them, *Salvia officinalis* L., i.e., "common sage" or "Dalmatian sage", is widely cultivated. These plants usually grow 30–70 cm tall, with a woody stem, whitish beneath and grayish-green above, and with purple-blue flowers up to 3 cm long appearing from early summer to early autumn [1]. Due to its worldwide spread, *S. officinalis* has been the most monitored species in relation to the biological potential of the whole plant as well as of its essential oils and polar extracts. For example, promising results were obtained in clinical studies with aqueous or ethanolic extracts of this *Salvia* species when focused on memory and cognitive functions, pain, and the biochemical profile of glucose and lipids [3]. In addition, in vivo assays in an ear edema induced by croton oil model pointed out the good anti-inflammatory activity of hydroethanolic extracts [4]. Indeed, in vitro experiments demonstrated that its ability to

inhibit 5-lipoxygenase activity [5] and to reduce the levels of interleukin 8 (IL-8) [6] might be based on these anti-inflammatory properties. Moreover, several authors also reported the benefits of *S. officinalis* polar extracts towards cell protection in distinct cell-based studies such as in HepG2, HeLa and Caco-2 cell lines, evidencing their possible usage as DNA-protective agents [7,8]. Notably, polar extracts from *S. officinalis* (aqueous, methanolic, ethanolic, and hydroalcoholic) have also been proven to have protective effects against oxidative events [9,10] or oxidative stress-related processes [11,12], as demonstrated in *in chemico*, cell-based or in vivo models [5,10–17].

In addition to *S. officinalis*, other widely distributed sage species such as *Salvia miltiorrhiza* Bge. and *Salvia hispanica* L. were highlighted by their richness in bioactive compounds and their potential health-promoting properties [1,2,18–20]. Still, many less-distributed species, including *Salvia greggii* A. Gray and *Salvia elegans* Vahl., remain poorly studied regardless of their broad use for culinary and medicinal purposes. *S. greggii*, also known as "autumn sage", is originated from Mexico and Texas, although it is currently spread in southwestern United States and Arizona and cultivated in some parts of the world. It grows as a soft, evergreen shrub taller than about 1.2 m, and, similarly to *S. officinalis*, its leaves are green and smooth [21]. Its flowers, which appear between spring to autumn, can be of different colors (red, pink, purple, white or orange) and are characterized by an intense aroma and abundant nectar. In turn, *Salvia elegans* is a species native of Mexico and is currently grown in the United States, Canada, and other regions of the world [22]. It grows as a sub-bush of 1–1.5 m high with a pineapple aroma and flavor, opposite leaves and oval, hairy, elliptic, pale green, and ruby red flowers [23].

S. greggii and *S. elegans* are widely used in traditional medicine, particularly in the form of infusions or decoctions, to treat digestive and oral problems (*S. greggii*) [1] or to lower blood pressure and combat central nervous system disorders for anxiety and insomnia (*S. elegans*) [23–25]. However, as far as we know, the phytochemical composition and beneficial effects of polar extracts, in particular those related to traditional usage (aqueous), remain unexplored. Regardless of this, *S. greggii* has been screened for terpenic compounds [26,27], and the antigerminative activity of its essential oils [28], while aspects related to polar extracts have not yet been studied. In turn, polar extracts from *S. elegans* have been the focus of some attention, and, in particular, hydroalcoholic extracts have been shown to exhibit antihypertensive, antidepressant, and anxiolytic effects [23–25] in in vitro models. Yet, to our knowledge, bioactive constituents of *S. elegans* polar extracts and their ability to counteract oxidative-stress-related events have not been previously elucidated.

Hence, the present study aimed to elucidate the phenolic composition and biological effects of *S. elegans* and *S. greggii* decoctions (mainly focusing on their potential antioxidant activity and inhibitory capacities towards key metabolic enzymes with impact in diabetes and obesity), while comparing the findings to those of the well-known *S. officinalis* species.

2. Results and Discussion

2.1. Phytochemical Composition

The decoction yields of the three *Salvia* species were approximately 20%, with slightly higher levels observed for *S. elegans* and *S. greggii* in comparison to *S. officinalis* (22.1 ± 2.2% and 22.2 ± 1.5% vs. 19.3 ± 2.3%, respectively). Consistent with their prevalence in *Salvia* plants [1,2,29], caffeic acid derivatives (particularly rosmarinic acid) were dominant compounds in *S. officinalis*, *S. elegans*, and *S. greggii* decoctions, accounting for about one third of the global identified phenolic species (Table 1, Figure 1, Figure S1). Nevertheless, significant differences could be found between extracts. *S. elegans* was distinguished by its richness in caffeic acid derivatives, namely rosmarinic acid (peak 36, $[M-H]^-$ at m/z 359→161, 179), caffeoylrosmarinic acid (peak 39, $[M-H]^-$ at m/z 537→493, 359) and salvianolic acid B (peak 27, $[M-H]^-$ at m/z 717→519), which overall represented approximately 70% of the total quantified phenolics, while the aqueous extract of *S. greggii* was characterized by high percentages of glycosidic flavones, mostly consisting of luteolin-*O*-hexoside and apigenin-*C*-hexoside

(peaks 24 and 20, $[M - H]^-$ at m/z 447 and 431, respectively), representing 33% and 20%, respectively, of total quantified phenolic compounds. Moderate amounts of luteolin-*C*-hexoside (peak 15, $[M - H]^-$ at m/z 447→327, 357), quercetin-*O*-hexoside (peak 22, $[M - H]^-$ at m/z 463→ 301) and two coumaric acid derivatives (peaks 6 and 8, $[M - H]^-$ at m/z 295 and 265, respectively) have also been detected in this extract. Interestingly, none of these compounds were detected in the decoctions of the two other species. Hence, globally, the extracts obtained from *S. elegans* and *S. greggii* species were clearly distinguishable from that of the *S. officinalis*, which was dominated by the *O*-hexuronic form of apigenin ($[M - H]^-$ at m/z 445→269, 48.4 ± 1.3 mg/g extract) and scutellarein ($[M - H]^-$ at m/z 461→285, 13.4 ± 0.6 mg/g extract), in addition to rosmarinic acid (28.3 ± 0.6 mg/g extract). Note that the predominance of apigenin-*O*-glucuronide and rosmarinic acid in this decoction is coherent with the abundance of these two constituents previously reported for polar extracts of this species (e.g., ethanol, methanol, aqueous, hydroalcoholic) [2,30]; however, it is worth noting that this is the first time that scutellarein-*O*-glucuronide was detected in *S. officinalis* extracts, while the previous studies only described its aglycone form.

Table 1. Phytochemical composition of *S. officinalis*, *S. elegans*, and *S. greggii* decoctions determined by UHPLC-DAD-ESI-MS^n.

NP	RT (min)	λmax	$[M-H]^-$	ESI-MS^2 Main Fragments	Compound	*S. off* *	*S. ele* *	*S. gre* *
1	1.5	275	149	103, 87, 131, 59	2,4-DimethylBA	4.1 ± 0.2	5.1 ± 0.1	7.3 ± 0.2
2	1.7	205	191	111, 173	Quinic acid	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.01
3	3.6	280	197	179, 73, 153	Danshensu	D	D	D
4	5.0	290, 324	353	191, 179, 135, 173	*cis* 3-*O*-CQA	-	-	3.1 ± 0.03
5	5.7	220, 278	137	109, 93, 119	HydroxyBA	D	2.0 ± 0.1	-
6	8.3	313	295	163	*p*-Coum Ac Pent	-	-	0.3 ± 0.02
7	8.8	290, 325	353	191, 179	*trans*-5-*O*-CQA	-	-	1.9 ± 0.1
8	9.4	313	265	177, 149, 119	Coumaric Ac Der	-	-	2.9 ± 0.05
		ND	325	163, 119	Caff Hex	D	-	-
9	9.7	290, 323	179	135	CaffAc	1.8 ± 0.04	1.5 ± 0.02	-
10	9.8	314	325	265, 235, 163	Coum Hex	D	-	-
11	9.9	255, 265, 350	625	463, 301	Querc diHex	-	-	D
12	12.1	271, 336	593	473, 503, 353	Api-6-*C*-Glc-7-*O*-Glc	4.3 ± 0.1	-	-
13	13.1	291, 311	637	351, 285, 193	Ferulic Ac Der	-	D	-
14	13.5	274	571	527, 483, 439, 373	YA E (isom1)	-	1.9 ± 0.1	-
15	13.9	256, 267, 345	447	327, 357	Lut-C-Hex	-	-	4.6 ± 0.09
16	13.9	281, 345	477	301, 373, 343, 397	Hydroxy-Lut-GlcA	D	1.9 ± 0.2	-
17	14.1	276	571	527, 439, 553, 483	YA E (isom2)	D	-	-
18	14.4	269, 304	473	311, 293, 179, 135	Cichoric acid	-	1.6 ± 0.07	-
19	14.8	267, 345	621	351, 269	Api-diGlcA	4.6 ± 0.3	-	-
20	15.2	268, 336	431	311, 341, 269	Api-*C*-Hex	-	-	15.7 ± 0.3
21	15.4	274	555	313, 357	SA K	1.6 ± 0.2	-	-
			571	527, 553, 509, 329	YA E (isom3)	D	-	-
22	15.8	255, 350	463	301	Querc-*O*-Hex	-	-	2.7 ± 0.2
23	15.9	280, 333	461	285	Scut-*O*-GlcA	13.4 ± 0.6	3.9 ± 0.1	-
24	16.0	255, 265, 348	447	285	Lut-7-*O*-Glc	-	-	26.1 ± 0.9
25	16.1	255, 266, 345	461	285	Lut-7-*O*-GlcA (isom1)	8.4 ± 0.3	5.1 ± 0.3	-
26	16.9	271, 306	521	359, 197, 179, 135	Salviaflaside	-	D	-
27	17.2	278	717	519, 475, 537, 339	SA B (isom1)	-	7.8 ± 0.4	-
28	17.3	279	571	527, 553, 329	YA E (isom4)	0.9 ± 0.1	-	-
29	17.7	279	717	537, 519, 339, 295	SA B (isom2)	-	1.7 ± 0.6	-
30	17.9	268, 334	577	269	Api-rut	4.5 ± 0.1	D	-
		283	719	359, 539, 521, 341	Sagerinic acid	6.0 ± 0.3	D	-
31	18.1	271, 304	717	519, 607, 339, 537	SA B (isom3)	-	1.7 ± 0.1	-
32	18.1	269, 329	431	269	Api-Hex	D	-	3.4 ± 0.2

Table 1. *Cont.*

NP	RT (min)	λmax	[M − H]⁻	ESI-MS² Main Fragments	Compound	*S. off* *	*S. ele* *	*S. gre* *
33	18.4	267, 337	445	269, 175	Api-GlcA	48.4 ± 1.3	3.2 ± 0.5	-
34	18.6	254, 266, 345	533	489, 447, 433	Lut malonyl Hex	-	-	D
35	18.6	270, 291, 326	717	555, 519, 475, 357	SA B (isom4)	D	-	-
36	19.0	218, 290, 328	359	161, 179, 197, 223	RA	28.3 ± 0.6	35.5 ± 0.8	10.9 ± 0.2
37	19.2	269, 307, 343	461	285	Lut-*O*-GlcA (isom2)	-	1.8 ± 0.1	
38	20.8	293, 328	373	343, 329, 311, 179	Methyl rosmarinate	-	-	D
39	21.2	290, 333	537	493, 359, 375	CaffRA/SA I (isom1)	1.2 ± 0.1	17.9 ± 0.1	-
40	21.4	293, 328	329	285, 314, 311, 161	CaffAc derivative	-	-	5.0 ± 0.03
		239, 285, 330	537	456, 493, 375, 359	CaffRA (isom2)	-	1.3 ± 0.04	-
41	22.1	295, 325	713	493, 359, 375	CaffAc der	-	D	-
42	22.3	280	537	456, 493, 359, 161	CaffRA (isom3)	-	0.7 ± 0.04	-
43	23.0	289, 327	717	519, 357, 555	SA B isomer	D	2.4 ± 0.04	-
					Caffeic acid and derivatives	39.8 ± 0.9	74.1 ± 0.5	20.8 ± 0.3
					Coumaric acid derivatives	-	-	3.2 ± 0.06
					Flavones	83.5 ± 2.3	15.9 ± 0.9	49.7 ± 1.3
					Flavonols	-	-	2.7 ± 0.2

NP—Number of peak represented in Figure 1; D—Detected; Ac—acid; Api- Apigenin; BA—Benzoic acid; CaffAc—Caffeic acid; Caff—Caffeoyl; CQA—Caffeoylquinic acid; Coum—Coumaroyl; Der—Derivative; Glc—Glucoside; GlcA—Glucuronide; Hex—Hexoside; Lut—Luteolin; Pent—Pentoside; Querc—Quercetin; Rut—Rutinoside; RA—Rosmarinic acid; SA—Salvianolic acid; *S. off*—*S. officinalis*; *S. ele*—*S. elegans*; *S. gre*—*S. greggii*; Scut—Scutellarein; YA—Yunnaneic acid; * values expressed as mg/g of extract.

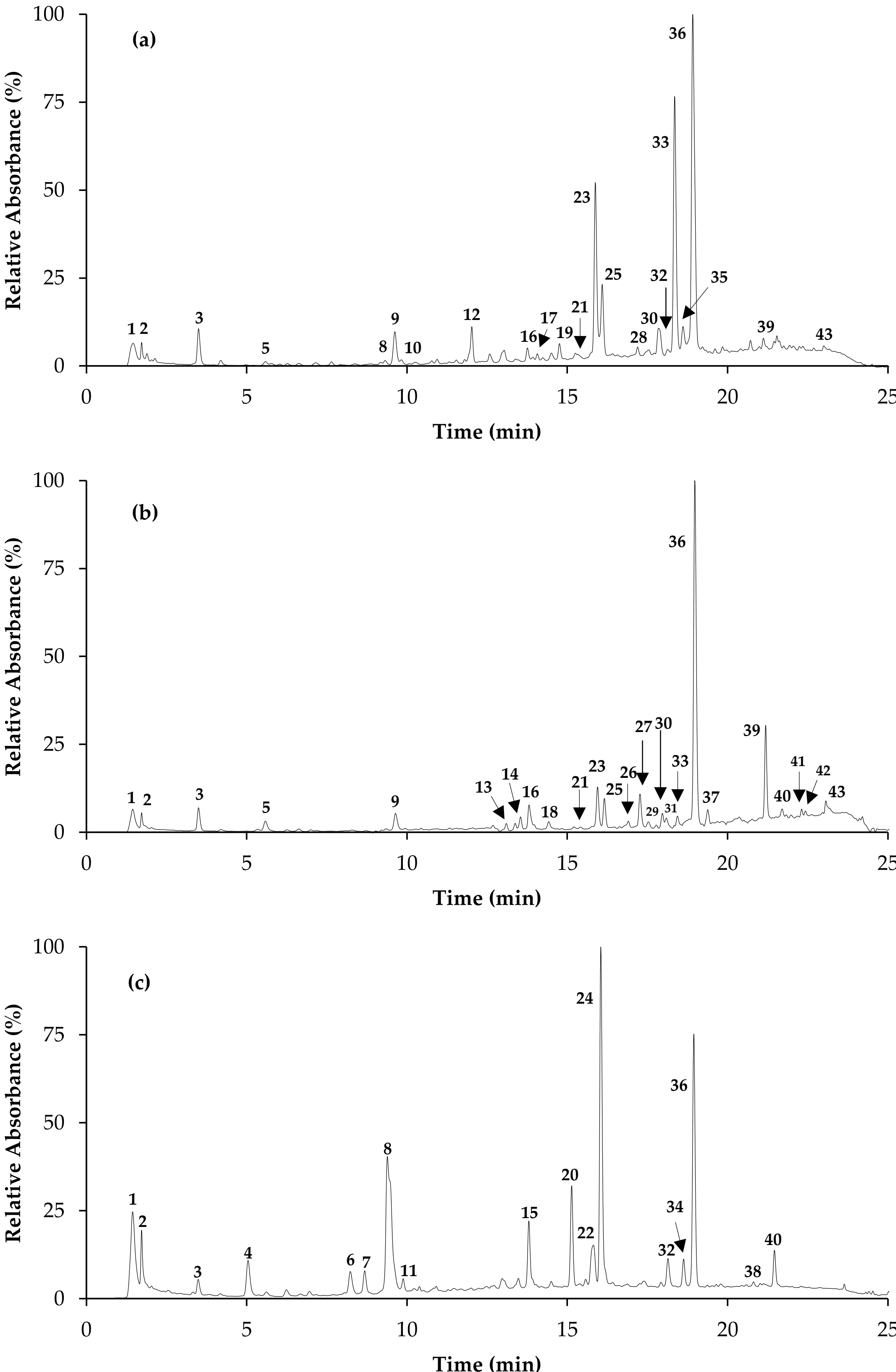

Figure 1. Chromatographic representation of *Salvia officinalis* (**a**), *Salvia elegans* (**b**), and *Salvia greggii* (**c**) decoctions at 280 nm. The numbers in the figure correspond to the UHPLC-DAD-ESI-MS^n peaks described in Table 1.

2.2. Biological Activities

2.2.1. Antioxidant Activity

The antioxidant ability of the aqueous extracts obtained from the *Salvia* plants were evaluated in regard to their ability to scavenge free radicals, namely DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), superoxide ($O_2^{\bullet-}$), nitric oxide (NO$^{\bullet}$), and peroxyl ($RO_2^{\bullet}$), and their capacity to reduce Fe^{3+} to Fe^{2+}. Furthermore, all the extracts were screened for their potency in inhibiting xanthine oxidase.

Globally, the *S. elegans* decoction was more promising than *S. greggii* in regard to its ability to scavenge free radicals and to reduce Fe^{3+} (Table 2). It presented EC_{50} values about 1.8–2.5 lower than the latter in DPPH$^{\bullet}$, NO$^{\bullet}$, $O_2^{\bullet-}$, and reducing power tests, and a tendentially higher ability to capture $RO_2^{\bullet}$. Notably, the *S. elegans* extract also presented tendentially better antioxidant potential than *S. officinalis*, with tendentially reduced EC_{50} values being registered for NO$^{\bullet}$, $O_2^{\bullet-}$, and reducing power tests, and even three times lower for the DPPH$^{\bullet}$ assay. In addition, one must highlight that the potency of *S. elegans* decoction to counteract DPPH$^{\bullet}$ and NO$^{\bullet}$ was 0.6- and 2.3-fold that of the ascorbic acid, respectively. The only exception was observed for the oxygen radical absorbance capacity (ORAC) assay for which the result observed for *S. officinalis* was better than that for *S. elegans*, although it was not statistically significant.

Table 2. Antioxidant properties of *S. officinalis*, *S. elegans*, and *S. greggii* decoctions.

	S. officinalis	***S. elegans***	***S. greggii***	**Standard**
DPPH$^{\bullet}$ (EC_{50} µg/mL) [(1)]	34.8 ± 3.3^{a}	10.7 ± 2.1^{b}	21.1 ± 2.5^{c}	6.69 ± 0.7^{b}
Reducing Power (EC_{50} µg/mL) [(2)]	40.0 ± 11.2^{a}	$31.3 \pm 5.0^{a,c}$	77.9 ± 5.6^{b}	16.30 ± 1.5^{c}
NO$^{\bullet}$ (EC_{50} µg/mL) [(1)]	118.2 ± 16.4^{a}	91.5 ± 14.5^{a}	167.8 ± 23.9^{b}	212.1 ± 9.7^{c}
$O_2^{\bullet-}$ (EC_{50} µg/mL) [(3)]	32.8 ± 0.6^{a}	30.6 ± 1.3^{a}	61.7 ± 3.4^{b}	7.8 ± 0.5^{c}
ORAC (µM TE/mg ext) [(4)]	404.4 ± 1.80^{a}	373.1 ± 28.1^{a}	335.6 ± 69.6^{a}	-
Xanthine oxidase (EC_{50} µg/mL) [(5)]	55.1 ± 10.6^{a}	71.8 ± 3.8^{b}	$70.1 \pm 4.0^{a,b}$	0.09 ± 0.01^{c}

[(1)] Ascorbic acid was used as the reference compound. [(2)] Amount of extract able to provide 0.5 of absorbance by reducing 3.5 µM Fe^{3+} to Fe^{2+}. Butylated hydroxyanisole (BHA) was used as a reference compound. [(3)] Gallic acid was used as the reference compound. [(4)] TE—Trolox Equivalent. [(5)] Allopurinol was used as the reference compound. Mean values ± SD; statistical analysis was performed by one-way ANOVA followed by Tukey's test. In each line, different letters mean significant differences ($p < 0.05$).

Table 3 summarizes the correlation coefficients between the amounts of classes of phenolic components found in the *Salvia* decoctions (caffeic acid and derivatives, coumaric acid derivatives, flavones and flavonols) and the data from the distinct biological experiments. According to these results, it is possible to suggest that the superior antioxidant activity of the *S. elegans* decoction is strongly associated with its richness in caffeic acid and derivatives, since correlation factors in DPPH$^{\bullet}$, reducing power, NO$^{\bullet}$, and $O_2^{\bullet-}$ assays were 0.801, 0.948, 0.986, and 0.844, respectively.

The comparison of the herein gathered data with that previously reported for other solvent-extracts or other *Salvia* species is not an easy task, since methodologic adaptations (e.g., radical precursor concentrations and their producing conditions) cause inevitable changes in EC_{50} values. This difficulty can be partly overcome by the comparison of the extract's potencies with that of reference compounds. Unfortunately, this approach is often not addressed by the authors. Moreover, there is no universal reference compound for a specific antioxidant assay, and variations in the selected standards are frequent within literature. Regardless of that, one must note that *S. officinalis* polar extracts have been commonly used as a reference for the assessment of antioxidant properties of other less-investigated plants [14,15], showing EC_{50} values in the range of 2.0 to 233.0 µg/mL for the DPPH$^{\bullet}$ assay [5,7,13–15,31]. Other ethanolic, methanolic, or aqueous extracts of *Salvia* origin, including those obtained from *Salvia amplexicaulis* [32], *Salvia ringens* [33], *Salvia verbenaca*, *S. sclarea* [34], *Salvia argentea* [15], and *Salvia nemorosa* [35], have been claimed to be good DPPH$^{\bullet}$ scavengers as well, with antioxidant potentials that, in some cases, equal those of the standard compounds (ascorbic acid,

butylated hydroxytoluene—BHT, or butylated hydroxyanisole—BHA). Hence, one might conclude that, in agreement with other studies reported for polar extracts of several *Salvia* species, *S. officinalis*, *S. elegans*, and *S. greggii* decoctions have a high ability to scavenge $DPPH^{\bullet}$, with *S. elegans* showing the most promising activity, followed by *S. greggii* and *S. officinalis.*

Table 3. Correlation coefficients between the amounts of phenolic components found in the *Salvia* decoctions (caffeic acid and derivatives, coumaric acid derivatives, flavones and flavonols) and the data from the distinct biological experiments.

	DPPH	RP	ORAC	NO	O2	XO	AG	L
Flavones	− 0.971	− 0.357	0.454	− 0.498	− 0.123	0.901	− 0.551	− 0.367
Flavonols	− 0.239	− 0.934	− 0.891	− 0.868	− 0.992	− 0.434	− 0.835	0.930
CafAcD	0.801	0.948	0.400	0.986	0.844	− 0.237	0.995	− 0.485
CouAcD	− 0.239	− 0.934	− 0.891	− 0.868	− 0.992	− 0.434	− 0.835	0.930
DPPH		0.570	− 0.228	0.690	0.356	− 0.771	0.734	0.134
RP			0.670	0.988	0.971	0.084	0.976	− 0.738
ORAC				0.547	0.829	0.796	0.493	− 0.995
NO					0.922	− 0.071	0.998 *	− 0.624
O2						0.321	0.996	− 0.878
XO							− 0.134	− 0.735
AG								− 0.574

Values expressed as Pearson correlation coefficient *R*; AG—α-glycosidase inhibitory activity; CafAcD—caffeic acid and derivatives; CouAcD—coumaric acid derivatives; DPPH—DPPH radical scavenging activity; L—lipase inhibitory activity; NO—nitric oxide radical scavenging capacity; ORAC—oxygen radical absorbance capacity; O2—superoxide anion scavenging activity; RP—reducing power potential; XO—xanthine oxidase inhibitory activity; * $p < 0.05$.

Polar extracts obtained from *Salvia* plants have also been previously screened for antioxidant abilities through other assays, although not as frequent as for $DPPH^{\bullet}$. In this context, Hamrouni-Sellami et al. [36] reported that the Fe^{3+} reducing ability of *S. officinalis* methanolic extracts was 6.5-fold less that of ascorbic acid, being in agreement with our results which also pointed to good effectiveness for decoctions of the same species. In general, our results also indicate that the three sage species herein studied possess promising $NO^{\bullet}$ scavenging capacities, as all the extracts had a lower EC_{50} compared to ascorbic acid. Moreover, their activity seems to be superior to that described by Chen and Kang [37] for the methanolic extracts of *Salvia plebeia* (EC_{50} = 216 ± 2.9 µg/mL), albeit that the absence of a reference compound in that study hampered solid conclusions. Furthermore, in our study, *S. elegans* and *S. officinalis* decoctions showed good $O_2^{\bullet-}$ scavenging capacity, also suggesting that these extracts might be more active than the methanolic extracts of *Salvia splendens* (EC_{50} = 527 µg/mL) [38]. Likewise, decoctions of *S. officinalis*, *S. elegans*, and *S. greggii* showed high capacity to scavenge $RO_2^{\bullet}$ (336–404 µM TE/mg), which was significantly superior to those previously reported for the aqueous and ethanolic extracts of *S. officinalis* (1143 and 2535 µM TE/g) and that other *Salvia* species (279–4735 µM TE/g).

Phenolic compounds have been previously reported to counteract the activity of xanthine oxidase (XO) [39,40], i.e., the enzyme that catalyzes the oxidation of hypoxanthine to xanthine, and further catalyzes xanthine to uric acid with a concomitant production of $O_2^{\bullet-}$, thus contributing to increment of oxidative stress events in cells. As can be observed in Table 2, the decoctions of the three *Salvia* species could effectively inhibit the activity of XO, albeit being less potent than the commercial drug allopurinol (EC_{50} = 55.1–71.8 µg/mL for *Salvia* extracts vs. 0.09 ± 0.01 µg/mL for allopurinol, respectively). Among the extracts, the most powerful was that from *S. officinalis*, a fact that could be related to its richness in apigenin glucuronide or in other flavones (i.e., scutellarein and luteolin glycosides), since these compounds have been described as strong inhibitors of this enzyme [41–44]. Although the individual effect of the compounds has not been tested by us, the correlation coefficients between the antioxidant assays and the main compounds of each aqueous extract are in good agreement (Table 3). In XO inhibitory assay, the flavones content of the *S. officinalis* decoction was highly correlated (0.901) with its XO inhibition capacity.

2.2.2. Metabolic Enzyme Activity

α-Glucosidase, α-amylase and pancreatic lipase are key digestive enzymes involved in the metabolism of carbohydrates and lipids, which make them important targets for therapeutic control of diabetes and obesity. α-Amylase and α-glucosidase catalyze the hydrolysis of carbohydrates into simple sugars, thus their inhibition retards the digestion of starch and oligosaccharides contributing to the reduction of postprandial increase in plasma glucose levels. In turn, lipase inhibition decreases the digestion of dietary triglycerides, hence reducing the levels of free fatty acids and monoacylglycerols in the intestinal lumen [40,45,46]. In this study, the ability of *S. officinalis*, *S. elegans*, and *S. greggii* decoctions to inhibit the activity of these three digestive enzymes were assessed through *in chemico* models.

Notably, the inhibitory activities of the three *Salvia* extracts against α-glucosidase were very promising, especially for *S. elegans* and *S. officinalis* (EC_{50} = 36.0 ± 2.7 μg/mL and 71.2 ± 5.0 μg/mL, respectively), demonstrating activities of 9- and 4-times that of the antidiabetic pharmaceutical drug, acarbose, respectively (Table 4). Moreover, despite being less effective than *S. elegans* and *S. officinalis*, *S. greggii* decoction was as effective as acarbose. Hence, our results suggest that the decoctions of *S. elegans*, *S. officinalis*, and *S. greggii* could serve as natural antidiabetic and anti-obesity agents to help in the control of glucose levels through the control of α-glucosidase activity. This hypothesis is also consistent with previous studies that reported identical results for polar extracts of *Salvia* against this enzyme, e.g., hydroethanolic extracts of S. *officinalis* (EC_{50} value of 69.7 μg/mL) [47] and methanolic extracts of *Salvia acetabulosa*, *S. nemorosa*, and *Salvia chloroleuca* (EC_{50} = 76.9 μg/mL, EC_{50} = 19 μg/mL and EC_{50} = 13.3 μg/mL, respectively) [35,48,49]. In vivo experiments have even demonstrated that the administration of a daily dose of *S. officinalis* methanolic extracts (500 mg/kg body weight) to alloxan-induced diabetic rats caused the inhibition of α-glucosidase activity comparable to that of the administration of acarbose (20 mg/kg bw) [45].

Table 4. Enzyme inhibitory properties of *S. officinalis*, *S. elegans*, and *S. greggii* decoctions.

	S. officinalis	*S. elegans*	*S. greggii*	Standard
α-Glucosidase (EC_{50} μg/mL) [(1)]	71.2 ± 5.0^{a}	36.0 ± 2.7^{b}	345.3 ± 6.4^{c}	357.8 ± 12.3^{c}
α-Amylase [(2)]	-	-	6.5 ± 3.0	0.7 ± 0.2
Pancreatic lipase [(3)]	4.6 ± 3.6^{a}	8.2 ± 0.3^{a}	14.4 ± 7.4^{a}	1.8 ± 0.4

[(1)] Acarbose was used as standard. [(2)] Results are expressed as percentage (%) inhibition at the concentration of 0.5 mg/mL (*Salvia* decoctions) or as EC_{50} (μg/mL), for the reference compound acarbose. [(3)] Results are expressed as percentage (%) inhibition at the concentration of 0.2 mg/mL (*Salvia* decoctions) or as EC_{50} (ng/mL), for the reference compound orlistat. In each line, different letters mean significant differences ($p < 0.05$).

The inhibitory capacity of polar extracts of *Salvia* species towards α-glucosidase have been mostly correlated with their phenolic constituents. In fact, Chen and Kang [37] reported that the inhibition of this enzyme by *S. plebeia* methanolic extracts increased proportionally to their total phenolic content. Moreover, Kocak et al. [50] reported that aqueous and methanolic extracts of *S. cadmica*, both rich in rosmarinic acid, luteolin, and apigenin, had high inhibitory effects towards α-glucosidase and α-amylase. Moreover, several phenolic compounds isolated from *S. miltiorrhiza*, namely, tanshinone IIA, rosmarinic acid, rosmarinic acid methyl ester and salvianolic acid C methyl ester, were reported to be stronger inhibitors of α-glucosidase than acarbose (EC_{50} = 0.042–0.23 μM and EC_{50} = 5.8 μM, respectively) [51]. The flavonoid compounds luteolin-7-*O*-glucoside, luteolin-7-*O*-glucuronide, and diosmetin-7-*O*-glucuronide, isolated from the aerial parts of *S. chloroleuca*, also showed potent α-glucosidase inhibitory effects with EC_{50} values of 18.3, 14.7, and 17.1 μM, respectively, exhibiting an inhibitory effect close to that of acarbose (EC_{50} = 16.1 μM) [49]. Note that rosmarinic acid and caffeoyl rosmarinic acid are two major phenolic components in *S. elegans* decoctions and, based on the mentioned bibliographic data, it is feasible to hypothesize that they might be important contributors for the higher inhibitory ability of this extract compared to the other two. In fact, correlation coefficients determined for the different assays have shown a strong correlation between the results obtained for

inhibitory activities on α-glucosidase and the content in caffeic acid and derivatives of the extracts (0.995). Interestingly, high correlation coefficients were also observed between the α-glucosidase and the antioxidant assays (0.976, 0.998, and 0.996 for reducing power, nitric oxide scavenging, and superoxide anion scavenging, respectively; Table 3), suggesting that metabolic and antioxidant effects might possibly be related.

However, regardless the great α-glucosidase inhibitory capacity and the fact that some authors have previously found potential inhibitory capacities in polar extracts of *Salvia*, namely for aqueous and methanolic extracts of *S. cadmica*, as well as for some individual phenolic compounds from *Salvia* origin [49], our results showed no substantial inhibition towards α-amylase up to the concentration of 0.5 mg/mL. Moreover, at 0.2 mg/mL, only *S. greggii* showed an anti-lipase activity higher than 10%. This could possibly be owed to its main phenolic component, i.e., luteolin-7-*O*-glucoside, since its aglycone has been reported to be a good lipase inhibitor [52,53], a hypothesis also supported by the high correlation found between the content of this flavone and the anti-lipase activity (0.930, data not shown). Interestingly, the anti-lipase activity of polar extracts of *Salvia* species has been previously described, namely for the methanolic extract of the leaves of *S. officinalis* (EC_{50} = 94 μg/mL) [54], the methanol extract of *Salvia spinosa* (EC_{50} = 156.2 μg/mL) [55], and methanol extracts of *Salvia triloba* (EC_{50} = 100.8 μg/mL) [56]. Hence, despite data from literature that seems to suggest that at least some polar extracts from *Salvia* species might be promising with respect to their abilities to control the activity of α-amylase and pancreatic lipase, this was not the observed for *S. officinalis*, *S. elegans* and *S. greggii* decoctions herein studied.

3. Materials and Methods

3.1. Chemicals

Ethanol, potassium di-hydrogen phosphate, and gallic acid were purchased from Panreac. Dimethylsulfoxide (DMSO), sodium chloride, potato starch, sodium and potassium tartrate, sodium hydroxide, and tris-HCl were purchased from Fisher (Pittsburgh, PA, USA). Fluorescein, 2,2′-azobis(2-amidinopropane)di- hydrochloride (AAPH), sodium nitroprusside, sulfanilamide, and 3,5-dinitrosalicylic acid (DNS) were purchased from Acros Organics (Hampton, NH, USA). Trolox, xanthine oxidase from bovine milk, allopurinol, α-glucosidase from *Saccharomyces cerevisiae*, 4-nitrophenyl α-D-glucopyranoside (pNPG), lipase from porcine pancreas and 4-nitrophenyl butyrate, α-amylase from porcine pancreas, β-nicotinamide adenine dinucleotide (β-NADH), phenazine methosulphate (PMS), nitrotetrazolium blue chloride (NBT), BHA (butylated hydroxyanisole), DPPH radical (2,2-diphenyl-2-picrylhydrazyl), ascorbic acid, and BHT (2,6-di-*tert*-butyl-4-methylphenol) were obtained from Sigma (St. Louis, MO, USA). Calcium chloride and sodium di-hydrogen phosphate were purchased from ChemLab (Eernegem, Belgium). Orlistat was purchased from TCI (Tokyo, Japan), acarbose from Fluka (Bucharest, Romania), xanthine from AlfaAesar (Ward Hill, MA, USA), and *N*-(1-naphthyl)ethylenediamine dihydrochloride from VWR (Radnor, PA, USA). Standard phenolics used for quantitative analysis were obtained from Extrasynthese. Folin-Ciocalteu reagent, Na_2CO_3, formic acid, and ethanol were purchased from Panreac (Barcelona, Spain). *n*-Hexane, methanol, and acetonitrile with high performance chromatography (HPLC) purity were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Direct-Q® water purification system (Merck Life Science, Darmstadt, Germany). All reagents were of analytical grade or of the highest available purity.

3.2. Plant Sampling and Preparation of Extracts

S. officinallis, *S. elegans*¸and *S. greggii* were purchased from Ervital (Viseu, Portugal) as a mixture of flowers and leaves, and stems where were cultivated under an organic regime. After collection, the aerial parts were dried in a ventilated incubator at 20 to 35 °C for 3 to 5 days.

Phenolic compounds were extracted by decoction according the method described by Ferreira et al. [57], with adaptations. A volume of 100 mL of distilled water was added to 5 g of

plant material (0.5 mm mesh powder) and the mixture was heated and then boiled for 15 min and filtered under reduced pressure through a G3 sintered plate filters. The resulting filtrated solution was concentrated in a rotary evaporator at 37 °C, followed by defatting with *n*-hexane (1:1 *v/v*). The resulting fraction was frozen, freeze-dried, and kept under vacuum in a desiccator in the dark for subsequent use [58].

3.3. Identification and Quantification of Phenolic Compounds

UHPLC-DAD-ESI/MS^n analyses of phenolic profiles from *S. officinalis*, *S. elegans*, and *S. greggii* decoctions (5 mg/mL) were carried out on an Ultimate 3000 (Dionex Co., San Jose, CA, USA) apparatus equipped with an ultimate 3000 Diode Array Detector (Dionex Co., San Jose, CA,, USA) and coupled to a Thermo LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA), an ion trap MS equipped with an electrospray ionization (ESI) source, following a method previous described [58]. Control and data acquisition were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific, San Jose, CA, USA). Nitrogen above 99% purity was used, and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with the ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. The full scan covered the mass range from *m/z* 100 to 2000. CID–MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with a collision energy of 25–35 arbitrary units.

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% (*v/v*) of formic acid in water, and solvent B consisted of acetonitrile, which was degassed and filtrated, using a 0.2 μm Nylon filter (Whatman International, Ltd., Maidstone, England) before use. The solvent gradient used consisted of a series of linear gradients starting from 5% of solvent B and increasing to 23% at 14.8 min, to 35% at 18 min, and to 100% at 21 min over three minutes, followed by a return to the initial conditions.

For quantitative determinations, the parameters of calibration curves, obtained by injection of known concentrations of the exact or structurally-related standard compounds, allowed the calculation of the limits of detection (LOD) and quantification (LOQ) [58].

3.4. Antioxidant Activities

3.4.1. $DPPH^•$ Scavenging Assay

Extracts capacity for scavenging $DPPH^•$ were evaluated following the procedure previously described by Catarino et al. [59]. Ascorbic acid was used as positive control. The concentration of the extract/standard able to scavenge 50% of $DPPH^•$ (EC_{50}) was determined using linear regression by plotting the percentage of inhibition against the concentration of the extracts.

3.4.2. Ferric Reducing Antioxidant Power (FRAP) Assay

For the reducing power assay, five different concentrations of each extract were prepared (0.05–0.25 mg/mL), and the assay was carried out according to a procedure described previously [59]. BHA was used as the positive control. A linear regression analysis was carried out by plotting the mean absorbance against the concentrations, and the EC_{50} value was determined considering the extract/standard concentration that provided 0.5 of absorbance.

3.4.3. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was performed according to the method previously described by Catarino et al. [60]. In a 96-well plate, 150 μL of fluorescein (10 nM), diluted from the stock solution of 250 μM, with 75 mM phosphate buffer (pH 7.4) were placed together with 25 μL of different trolox concentrations (3.13–25 μM). The same process was repeated for the extracts with final concentrations ranging between 0.4–6.3 μg/mL. For blanks, 25 μL of phosphate buffer was added instead of antioxidant solutions. After 10 min incubation at 37 °C, 25 μL of 2,2′-azobisisobutyramidinium chloride (AAPH) (153 mM)

solution was added to each well to reach a final reaction volume of 200 µL. The plate was immediately placed in the plate reader (Biotek, Austria), and fluorescence was monitored every minute over 60 min. The measurement was carried out at 37 °C with automatic agitation for 5 s prior to each reading. Excitation was conducted at 485 nm with a 20 nm bandpass, and emission was measured at 528 nm with a 20 nm bandpass. Six concentration-dependent kinetic curves were obtained for each sample and for trolox as well. The area under the curve (AUC) of the fluorescence decay and Net AUC were calculated according to the following equations (1–3):

$$\mathrm{AUC} = 1 + \sum_{t_0\,=\,60\ \mathrm{min}}^{t_i\,=\,60\ \mathrm{min}} \frac{R_i}{R_0}, \quad (1)$$

$$\text{Net AUC} = \mathrm{AUC}_{\text{sample}} - \mathrm{AUC}_{\text{blank}}, \quad (2)$$

where R_0 is the fluorescence reading at the initiation of the reaction and R_i is the fluorescence reading at the time i. Antioxidant activities (ORAC values) of the extracts were calculated by using the following ratio:

$$\text{ORAC value} = \frac{m_e}{m_T}, \quad (3)$$

where m_e is the slope of the curve of Net AUC vs. extract concentrations, and m_T is the slope of the curve of Net AUC vs. trolox concentrations. The final results were expressed in µM of trolox equivalents (µM TE) per µg of sample extract.

3.4.4. $NO^{\bullet}$ Scavenging Assay

The $NO^{\bullet}$ scavenging method was adapted from Catarino et al. [60]. Briefly, 100 µL of six different extract concentrations (0–1 mg/mL) were mixed with 100 µL of sodium nitroprusside (3.33 mM in 100 mM sodium phosphate buffer pH 7.4) and incubated for 15 min under a fluorescent lamp (Tryun 26 W). Afterwards, 100 µL of Griess reagent (0.5% sulphanilamide and 0.05% naphthyletylenediamine dihydrochloride in 2.5% H_3PO_4) were added to the mixture, which was allowed to react for another 10 min in the dark. The absorbance was then measured at 562 nm, and the percentage of $NO^{\bullet}$ scavenging was calculated using the equation described by Yen and Der Duh [61] as follows:

$$\%\ NO^{\bullet}\ \text{scavenging} = \frac{A_c - A_e}{A_c} \times 100, \quad (4)$$

where A_c is the absorbance of the control (without extract addition) and A_e is the absorbance of the extract. Ascorbic acid was used as the reference compound. The concentration of the extract/standard able to scavenge 50% of $NO^{\bullet}$ (EC_{50}) was then calculated by plotting the percentage of inhibition against the extract concentrations.

3.4.5. Superoxide Anion ($O_2^{\bullet-}$) Scavenging Assay

The $O_2^{\bullet-}$ scavenging method was carried out according to the method described by Catarino et al. [60]. Briefly, in a 96-well plate, 75 µL of six different sample concentrations (0.0–250 µg/mL) were mixed with 100 µL of β-NADH (300 µM), 75 µL of NBT (200 µM), and 50 µL of PMS (15 µM). After 5 min, the absorbances at 560 nm were recorded and the scavenging activity of superoxide radicals was calculated according to Equation (4). Gallic acid was used as the reference compound. The concentration of the extract/standard able to scavenge 50% of $O_2^{\bullet-}$ (EC_{50}) was determined using linear regression by plotting the percentage of inhibition against the concentration of the extracts.

3.4.6. Inhibition of Xanthine Oxidase Activity

Inhibition of xanthine oxidase activity was carried out following the method described by Filha et al. [62], with slight modifications. Briefly, in a 96-well plate, 40 µL of extract concentrations

(0–2 mg/mL) were mixed with 45 μL of sodium dihydrogen phosphate buffer (100 mM, pH 7.5) and 40 μL of enzyme (5 mU/mL). After 5 min incubation at 25 °C, the reaction was started with the addition of 125 μL of xanthine (0.1 mM dissolved in buffer) and the absorbance at 295 nm was measured every 45 s over 10 min at 25 °C. The inhibitory effects towards xanthine oxidase activity was calculated as follows:

$$\% \text{ inhibiton} = \frac{m_c - m_e}{m_c} \times 100, \tag{5}$$

where m_c is the slope of the straight-line portion of the curve generated by the control (no inhibitor) and m_e is the slope of the straight-line portion of the curve generated by each extract. Allopurinol was used as a positive control of inhibition. The concentration of the extract/standard able to inhibit 50% (EC_{50}) of the activity of the enzyme was determined using linear regression by plotting the percentage of inhibition against the concentration of the extracts.

3.5. Inhibition of Enzymatic Activities

3.5.1. Inhibition of α-Glucosidase Activity

Inhibition of α-glucosidase activity was measured following the method described by Neto et al. [63], with slight modifications. In short, 50 μL of different extract concentrations (0–2 mg/mL, in 50 mM phosphate buffer pH 6.8) were mixed with 50 μL of 6 mM 4-nitrophenyl α-D-glucopyranoside (pNPG), dissolved in deionized water. The reaction was started with the addition of 100 μL of α-glucosidase solution, and the absorbance was monitored at 405 nm every 60 s for 20 min at 37 °C. The inhibitory effects towards α-glucosidase activity was calculated as in Equation (5). Acarbose was used as a positive control of inhibition. The concentration of the extract/standard able to inhibit 50% (EC_{50}) of the activity of the enzyme was determined using linear regression by plotting the percentage of inhibition against the concentration of the extracts.

3.5.2. Inhibition of α-Amylase Activity

Inhibition of α-amylase activity was measured according to Wickramaratne et al. [64], with slight modifications. Briefly, 200 μL of extract six different extract concentrations (0–2 mg/mL) dissolved in 20 mM phosphate buffer (pH 6.9, containing 6 mM of NaCl) were added to 400 μL of a 0.8% (w/v) starch solution in the same phosphate buffer, and the mixture was incubated for 5 min at 37 °C. The reaction was then started with the addition of 200 μL of α-amylase solution, and after 5 min of incubation, 200 μL of the reaction mixture was collected and immediately mixed with 600 μL of DNS reagent (10 g/L of 3,5-dinitrosalicylic acid, 300 g/L of potassium and sodium tartrate tetrahydrate, and 0.4 M NaOH) to stop the reaction. A second aliquot of 200 μL was further collected 15 min later and mixed with DNS reagent as well. Samples were then boiled for 10 min, and, once they had cooled, 250 μL were transferred to each well in a 96-well microplate for absorbance reading at 450 nm. Blank readings (no enzyme) were then subtracted from each well and the inhibitory effects towards α-amylase activity was calculated as follows:

$$\% \ inhibiton = \frac{\Delta Abs_c - \Delta Abs_e}{\Delta Abs_c} \times 100, \tag{6}$$

where ΔAbs_c is the variation in the absorbance of the negative control and ΔAbs_e is the variation in the absorbance of the extract. Acarbose was used as a positive control of inhibition.

3.5.3. Inhibition of Pancreatic Lipase Activity

The lipase activity was measured according to the procedure described by Neto et al. [63], with slight modifications. The reaction mixture was prepared in a microtube by mixing 55 μL of five different concentrations of extract (0–2 mg/mL) dissolved in tris buffer 100 mM (pH 7.0) with 467.5 μL of tris-HCl (100 mM, pH 7.0, containing 5 mM of $CaCl_2$) and 16.5 μL of enzyme. The reaction was

started by adding 11 μL of 20 mM 4-nitrophenyl butyrate diluted in DMSO. Final DMSO concentration in the reaction mixture did not exceed 2%. The reaction mixture was then quickly transferred to a 96-well plate and incubated for 35 min at 37 °C while the absorbance was being measured every 60 s at 410 nm. The inhibitory effects towards pancreatic lipase activity was calculated as in Equation (5). Orlistat was used as a positive control of inhibition.

3.6. Statistical Analysis

All data are presented as mean ± standard deviations from three independent assays performed at least in duplicate. One-way analysis of variance (ANOVA) followed by Tukey´s test was used to detect any significant differences among different means. Correlation analyses were performed using a two-tailed Pearson's correlation test. A p-value less than 0.05 was assumed as significant. The results were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and SPSS v 23.0 (Statistical Package for the Social Sciences).

4. Conclusions

This work clarifies the antioxidant properties of *S. elegans*, *S. greggii*, and *S. officinalis* decoctions as well as their inhibition towards the activity of carbohydrate and lipid metabolic enzymes, highlighting possible correlations with their phenolic components. It was shown that among the three plants, *S. elegans* decoctions were the most promising regarding antioxidant activity and inhibitory potential against α-glucosidase, a fact that might be related to its richness in caffeic acid and its derivatives. In turn, despite all the three decoctions of *Salvia* species could effectively inhibit the activity of xanthine oxidase, one should highlight the superior inhibitory capacity of *S. officinalis*, which is possibly associated with the presence of flavones. In conclusion, similarly to the well-known *S. officinalis* species, *S. elegans* and *S. greggii* are a valuable source of natural metabolites and could be used for commercial applications in novel functional foods or pharmaceutical ingredients targeting diabetes and obesity prevention.

Supplementary Materials: Supplementary Materials can be found in a separate file: Figure S1: UV spectra of the main peaks identified in *S. elegans*, *S. greggii*, and *S. officinalis* decoctions.

Author Contributions: M.D.C. performed the experimental work and co-wrote the manuscript; O.R.P. and A.F.A. performed the analysis and interpretation of data and wrote the manuscript; A.M.S.S. co-wrote the manuscript; S.M.C. coordinated the work and co-wrote the manuscript.

Funding: The Science and Technology Foundation/Ministry of Education and Science (FCT/MEC) funded the Organic Chemistry, Natural Products and Food Stuffs Research Unit (QOPNA) research unit (FCT UID/QUI/00062/2013) through national funds and, where applicable, was co-financed by the European Regional Development Fund (FEDER), within the Portugal 2020. Project AgroForWealth (CENTRO-01-0145-FEDER-000001), funded by Centro2020, through FEDER and PT2020, financed the research contract of Susana M. Cardoso. The Science and Technology Foundation financed Marcelo D. Catarino (fellowship PD/BD/114577/2016).

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of extracts/compounds are available from the authors.

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Beneficios para la salud y perfil fitoquímico de decocciones de *Salvia apiana* y *Salvia farinacea* var. *victoria blue*

Objetivos

El presente estudio tiene como objetivo investigar los posibles efectos biológicos de las decocciones de *S. apiana* y *S. farinacea* var. *victoria blue*, relacionadas con sus actividades antioxidantes, antiinflamatorias, citotoxicidad y antibacterianas, asociando estos efectos con los compuestos fenólicos presentes en sus extractos.

1. Introducción

El estrés oxidativo surge cuando se rompe el equilibrio entre la formación de especies reactivas y su eliminación, produciéndose la acumulación de estos compuestos, que causan varios daños celulares. Esta situación está asociada con el envejecimiento y la aparición de diversas enfermedades, incluido el cáncer. En este contexto, se cree que los suplementos dietéticos con productos de origen vegetal ricos en fitoquímicos pueden contrarrestar los eventos relacionados con la oxidación, aportando beneficios para la salud [1,2].

Salvia es el género mayor entre las plantas de la familia Lamiaceae, siendo conocidas por su riqueza en aceites esenciales y/o compuestos fenólicos [3,4]. *Salvia apiana* y *Salvia farinacea var. victoria blue* son dos plantas originarias de las regiones del suroeste de los Estados Unidos y México. La primera también se conoce como salvia blanca, es un arbusto ramificado con hojas blancas y plateadas, muy aromáticas y flores blancas o de color lavanda pálida. *Salvia farinacea var. victoria blue* es un arbusto perenne, con hojas de color verde, irregularmente aserradas, y contiene unas puntas de flores de color violeta-azul abundantes y compactas, de múltiples ramas, que se asemejan a la lavanda [5–7]. *S. apiana* se ha estudiado previamente con respecto a la composición de sus aceites esenciales y extractos, que tienen altas cantidades de terpenos y sus derivados [8,9]. Con respecto a la *S. farinacea* var. *victoria blue*, las propiedades químicas y biológicas de los extractos polares permanecen inexploradas.

2. Métodos

2.1. Plantas y extracción de compuestos fenólicos

Las partes aéreas de las plantas *S. apiana* y *S. farinacea* var. *victoria blue* se cultivaron bajo un régimen orgánico, se cosecharon y se secaron durante 3 a 5 días. Los extractos fenólicos se han obtenido utilizando una decocción en la proporción 1:20. Seguidamente

las soluciones se filtraron, se concentraron, se evaporaron, se congelaron a -20°C y se liofilizaron. Se obtuvieron tres extractos acuosos de cada planta.

2.2 Identificación y cuantificación de compuestos fenólicos

Los extractos fueron caracterizados y cuantificados por cromatografía líquida de alta eficiencia, asociada a espectrometría de masas por electrospray en el modo de ionización negativo (UHPLC-DAD-ESI/ MS^n).

2.3 Pruebas de bioactividad

2.3.1 Capacidad antioxidante

La actividad antioxidante de los extractos acuosos de *S. apiana* y *S. farinacea* var. *victoria blue* se evaluó mediante cuatro ensayos químicos. Se hicieron las pruebas de radicales 2,2-difenil-1-picrilhidrazil ($DPPH^{\bullet}$) y del poder reductor por la reducción de hierro (III) [10], usando como estándar ácido ascórbico y hidroxianisol butilado (BHA), respectivamente. Se evaluó también la inhibición de la peroxidación de lípidos por la prueba del blanqueo del β-caroteno y la prueba de la disminución de las sustancias reactivas al ácido tiobarbitúrico (TBARS) [11], usando en ambos ensayos Trolox como control positivo. Los resultados se expresaron como valores de EC_{50} (concentración de la muestra que proporciona el 50% de la actividad antioxidante) en µg por ml de extracto.

2.3.2 Capacidad anti-inflamatoria

La actividad antiinflamatoria de los extractos de *Salvia* se determinó mediante un modelo celular, evaluando la inhibición de la producción de NO en líneas celulares de macrófagos RAW 264.7 después de la estimulación con LPS (lipopolisacárido) [12]. Los resultados se expresaron como EC_{50} (µg/mL), que corresponde a la concentración de la muestra que proporciona 50% de inhibición de la producción de NO. La dexametasona se utilizó como control positivo mientras que los controles negativos no tenían LPS.

2.3.3 Citotoxicidad en células tumorales y no tumorales

El efecto citotóxico de los extractos de *S. apiana* y *S. farinacea var. victoria blue* se llevaron a cabo mediante el ensayo de sulforodamina B (SRB) [12], en cuatro líneas celulares de tumores humanos MCF-7 (adenocarcinoma de mama), NCI-H460 (cáncer de células del pulmón), HeLa (carcinoma cervical) y HepG2 (carcinoma hepatocelular). Además, la citotoxicidad de los extractos de *Salvia* se analizó mediante un ensayo de hepatotoxicidad, en células primarias no tumorales de hígado de cerdo (PLP2). Los resultados de la citotoxicidad se expresaron como valores GI_{50} (µg/mL), correspondientes a la concentración de la muestra que inhibió el 50% del crecimiento celular. En estos experimentos, se utilizó elipticina como control positivo.

2.3.4 Capacidad anti-bacteriana

El potencial antibacteriano de los extractos polares de las dos plantas de *Salvia* se evaluó frente a cinco cepas bacterianas, incluyendo dos Gram positivas (Gram+)

Staphylococcus epidermidis y *Staphylococcus aureus*, y tres Gram negativas (Gram-) *Salmonella typhimurium, Escherichia coli* y *Pseudomonas aeruginosa*, por el método de microdilución en el medio, calculándose la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM). La nisina se utilizó como control positivo.

3. Resultados y discusión

3.1. Actividad antioxidante

En general, la decocción de *S. apiana* presentó una capacidad antioxidante superior a la de *S. farinacea* var. *victoria blue*, una tendencia que fue particularmente evidente con respecto a los eventos de peroxidación lipídica (Tabla 1), con valores de EC_{50} alrededor de ocho veces menor o igual que el estándar comercial, en los ensayos de blanqueo del β-caroteno y TBARS, respectivamente. A pesar de ser menos activa, *S. farinacea* var. *victoria blue* también se puede considerar como un agente antioxidante prometedor, ya que los valores de EC_{50} en todas las pruebas fueron solo 1.8-3.7 veces mayores que los de los compuestos estándar.

Tabla 1 – Actividad antioxidante (EC_{50}, μg/mL) de decoctions de *S. apiana* y *S. farinacea* var. *victoria blue*

Prueba	***S. apiana***	***S. farinacea* var. *victoria blue***	**Estándar**		
			AA	BHA	Trolox
DPPH$^{\bullet}$	13.3±1.1^{a}	17.4±5.5^{a}	6.7±0.7^{b}		
Poder Reductor	55.0±5.6^{a}	59.9±3.6^{a}		16.1±2.0^{b}	
TBARS	2.79±0.1^{a}	42.2±0.6^{b}			23.0±1.0^{c}
β-carotene bleaching inhibition	41.2±1.6^{a}	153.5±2.2^{b}			41.7±0.3^{a}

AA – Ácido ascórbico; BHA - hidroxianisol butilado; DPPH$^{\bullet}$ - 2,2-difenil-1-picrilhidrazil; TBARS - sustancias reactivas al ácido tiobarbitúrico. Valores medios ± SD. El análisis estadístico se realizó mediante ANOVA de una vía, seguido de la prueba de Tukey. En cada fila, letras diferentes significan diferencias estadísticas significativas ($p < 0.05$).

Las capacidades antioxidantes de los extractos polares de *S. apiana* y *S. farinacea* var. *victoria blue* no han sido explorados anteriormente. Sin embargo, las prometedoras habilidades descritas en este estudio para estas decocciones están de acuerdo o incluso, y en particular en el caso de *S. apiana*, han excedido las descritas anteriormente para plantas de salvia [13–15].

3.2. Actividad antiinflamatoria

Se ha descrito que el óxido nítrico producido en altas concentraciones por los macrófagos está involucrado en la inflamación y los compuestos dirigidos a su producción se consideran buenos candidatos para atenuar las enfermedades relacionadas con la inflamación [16]. La potencia de las decocciones de *S. apiana* y *S.*

farinacea var. *victoria blue* para contrarrestar la producción de NO• por macrófagos RAW 264.7 activados por LPS, se muestra en la Tabla 2. Ambos extractos fueron efectivos, y particularmente el de *S. apiana* fue bastante prometedor, ya que su potencia (EC_{50}=49.9 µg/mL) equivalía a aproximadamente un tercio de la de dexametasona (EC_{50}=16.0 µg/mL).

Tabla 2 – Actividad antiinflamatoria (EC_{50}, µg/mL) y efecto citotóxico (GI_{50}, µg/mL) de extractos acuosos de *S. apiana* y *S. farinacea* var. *victoria blue*

	S. apiana	***S. farinacea* var. *victoria blue***	**Estándar**
Actividad antiinflamatoria			Dexametasona
NO• production	49.9±2.5[a]	80.8±0.4[b]	16.0±1.0 [c]
Citotoxicidad hacía células tumorales			Elipticina
HepG2 (carcinoma hepatocelular)	40.9±3.3[a]	87.4±5.4[b]	1.0±0.2[c]
HeLa (carcinoma cervical)	57.3±5.1[a]	77.8±3.5[b]	2.0±0.1[c]
MCF-7 (adenocarcinoma de mama)	60.2±4.2[a]	59.8±0.1[a]	1.00±0.04[b]
NCI-H460 (cáncer de células del pulmón)	245.7±6.3[a]	279.5±10.1[b]	1.0±0.1[c]
Citotoxicidad hacía células no tumorales			
Inhibición de PLP2	361.7±5.3[a]	335.4±8.0[b]	3.0±1.0[c]

Los medios seguidos por las mismas letras en cada fila no difieren según la prueba estadística de Tukey ($p < 0.05$)

Además, su actividad fue superior a la descrita anteriormente para extractos polares de *Salvia miltiorrhiza* [17] y *Salvia plebeia* [18] (EC_{50} de 200 y ≥1000 µg/mL, respectivamente), aunque los autores no establecieron ninguna relación entre los efectos antiinflamatorios y los posibles compuestos involucrados.

3.3 Efecto citotóxico en células tumorales y no tumorales

Las plantas medicinales, incluidas las especies de *Salvia*, se han estudiado ampliamente como fuentes potenciales de agentes quimioterapéuticos para el cáncer, particularmente en modelos celulares *in vitro* [19,20]. En general, las decocciones *S. apiana* y *S. farinacea* var. *victoria blue* presentaron efecto citotóxico hacía células tumorales, en particular células del adenocarcinoma de mama MCF-7, carcinoma cervical HeLa y carcinoma hepatocelular HepG2 (Tabla 2). Entre los dos extractos, *S. apiana* exhibió más citotoxicidad que la de *S. farinacea* var. *victoria blue*, un hecho que quedó claro en las células HepG2 (GI_{50} de 40.9 vs 87.4 µg/mL, respectivamente) y HeLa (GI_{50} de 57.3 vs 77.8 µg/mL, respectivamente). En un estudio previo, Saeed et al. [21]

encontraron un elevado efecto citotóxico de los extractos metanólicos de *S. apiana* contra células de leucemia sensibles y resistentes a múltiples fármacos (valores GI_{50} de 7.17±0.67 y 9.91±0.80 µg/mL, respectivamente). Además, aunque nuestros resultados no pueden compararse directamente con los publicados anteriormente para otras plantas, debido a las diferencias en las metodologías y los estándares empleados, apoyan el concepto de que los extractos polares de plantas de *Salvia* pueden actuar como agentes citotóxicos. De hecho, otros autores han descrito previamente los efectos citotóxicos para extractos polares de otras especies de *Salvia*, a saber, *Salvia eremophila*, que fue efectiva contra células del carcinoma de mama MCF-7 [19], o raíces de *S. miltiorrhiza* y *Salvia officinalis* [20] con actividad citotóxica frente a células de carcinoma hepatocelular HepG2, que presentaron en general buenos valores de GI_{50} (rango 17 y 83 µg/ml).
Además, nuestros resultados también indicaron que las decocciones de *S. apiana* y *S. farinacea* var. *victoria blue* tienem capacidad de discriminar las células tumorales, lo que es esencial para minimizar los efectos citotóxicos en las células normales, como se refleja en valores de GI_{50} bajos en las líneas celulares de cáncer en comparación con los de las células PLP2 (GI_{50} = 361.7 y 335.4 µg/mL, respectivamente).

3.4. Actividad antibacteriana

Las potencialidades de compuestos de plantas, incluyendo *Salvias,* como agentes antimicrobianos, evidencian sus aplicaciones potenciales en la conservación de alimentos en la industria alimentaria, así como en el desarrollo de nuevos fármacos para el tratamiento de enfermedades infecciosas [7,22].

Tabla 3 – CIM (mg/mL) y CBM (mg/mL) de extractos de *S. apiana* y *S. farinacea* var. *victoria blue* y nisina (mg/mL) hacía bacterias seleccionadas

	S. apiana		***S. farinacea* var. *victoria blue***		**Nisina**	
Bacteria	**CBM**	**CIM**	**CBM**	**CIM**	**CBM**	**CIM**
Gram+						
S. epidermidis	0.69	0.34	8.50	8.50	<0.63	<0.63
S. aureus	0.69	0.69	2.12	1.06	<0.63	<0.63
Gram-						
S. tyiphimurium	2.75	2.75	>8.5	>8.5	0.5	0.5
E. coli	2.75	2.75	8.5	8.5	1.0	0.5
P. aeruginosa	2.75	2.75	>8.5	>8.5	1.0	1.0

CIM - concentración inhibitoria mínima; CBM - concentración bactericida mínima

El potencial antibacteriano de las dos decocciones de *Salvia* hacia las cinco bacterias se resume en la Tabla 3. En general, *S. apiana* mostró que tenía propiedades antibacterianas prometedoras, con capacidad inhibitoria y letal en concentraciones iguales o inferiores a 0.69 mg/ml (para *S. aureus* y *S. epidermidis*) y de 2.75 mg/ml (para el *S. typhimurium, E. coli* y *P. aeruginosa*), que correspondían a solo 3 a 5 veces menos que la nisina.

De una manera diferente, *S. farinacea* var. *victoria blue* mostró un efecto antibacteriano relevante contra la bacteria Gram+ *S. aureus* (MIC y MBC de 1.06 y 2.12 mg/ml, respectivamente), aunque se requería una mayor concentración (CMI y MBC de 8.5 mg/ml) para tener un efecto bactericida contra *S. epidermidis* y *E. coli.*

Además, esta planta no mostró ninguna actividad inhibitoria o letal contra las bacterias Gram- *S. typhimurium* y *P. aeruginosa.* Poco se sabe sobre los efectos antibacterianos de estas especies de *Salvia*, aunque existen resultados prometedores en otras especies, en algunos casos asociados con su riqueza en compuestos fenólicos. Por ejemplo, *Salvia bicolor* mostró actividad poderosa contra bacterias Gram+ y Gram- (valores de CIM en el rango de 0,2 a 1 mg/mL), cuyo efecto antibacteriano fue asociado a su riqueza en compuestos, que eran en su mayoría ácidos gálico, *p*-cumárico y protocatecúico, y flavonoides (por ejemplo, luteolina-7-*O*-glucósido) [23].

3.5. Caracterización de los extractos

Los niveles de compuestos fenólicos en las decocciones de *Salvia* fueron significativamente diferentes, con valores aproximadamente seis veces más elevados en *S. apiana* que en *S. farinacea* var. *victoria blue* (Tabla 4). Además de su riqueza en ácido rosmarínico (56.8 y 17.8 mg/g de extracto, respectivamente), como es habitual en los extractos polares de plantas de *Salvia*, los dos extractos difirieron con respecto a sus constituyentes fenólicos, así como a los descritos previamente para la decocciones de *Salvia elegans*, *Salvia greggii* y *Salvia officinalis* [24]. De hecho, la decocción de *S. apiana* se caracterizó por su riqueza en derivados de terpenos, que estaban representados principalmente por el rosmanol, un derivado de sageone y ácido hidroxicarnosico, con niveles estimados de 192.4, 174.1 y 69,7 mg/g de extracto, respectivamente (Tabla 4). *S. apiana* también contenía otros compuestos menos abundantes, como carnosol, ácido carnósico y algunos flavonoides (hesperidina, quercetina-*O*-hexósido y cirsimaritina). Estos resultados están de acuerdo con los publicados previamente [9,25,26], permitiendo concluir que los extractos polares de *S. apiana* son particularmente ricos en terpenos fenólicos.

Aunque menos ricos en compuestos fenólicos, además del ácido rosmarínico, la decocción de *S. farinacea* var. *victoria blue* presentó cantidades moderadas de flavonas

comunes a las plantas de *Salvia* [27–29], como la apigenina-*O*-hexósido (fracción 28, 16.7 mg/g de extracto) y la luteolina-*O*-glucurónido (fracción 24, 15.8 mg/g de extracto).

Tabla 4 – Identificación y cuantificación de los compuestos identificados en las decocciones de *S. apiana* y *S. farinacea* var. *victoria blue* por UHPLC-DAD-ESI-MS^n.

Fr	TR	UV max	$[M-H]^-$	Compuesto probable	*S. apiana**	*S. farinacea* var. *victoria blue**
1	1.5	275	149	Ácido dimetil benzoico	-	5.9±0.1
2	1.7	205	191	Ácido quinico	5.0±0.3	0.40±0.01
3	3.6	280	197	Danshensu	D	D
4	4.4	261, 289	153	Ácido protocatechuico	D	-
5	5.1	290, 324	353	*cis* 3-*O*- Ácido cafeioilquinico	D	-
6	5.4	294, 322	353	*trans* 3-*O*- Ácido cafeioilquinico	-	D
7	7.9	309	337	Ácido cumarico quinico	D	0.30±0.03
8	8.3	313	295	*p*- Ácido cumarico pentosido	0.40±0.04	-
9	8.8	290, 325	353	*trans* 5-*O*- Ácido cafeioilquinico	-	0.60±0.03
10	9.5	290, 325	353	4-*O*- Ácido cafeiiloquinico	5.5±0.1	-
11	9.7	290, 323	179	Ácido cafeico	-	0.8±0.0
12	9.8	314	325	Ácido cumarico hexósido	-	0.3±0.0
13	11.8	311	337	Ácido cumarico quinico	D	0.2±0.0
14	12.8	287, 324	367	Ácido feruloil quínico	-	D
15	13.0	309	225	Derivado del ácido cumaroico	1.8±0.1	-
16	13.1	291, 311	637	Derivado del ácido ferúlico	-	0.5±0.0
17	13.5	274	571	Ácido yunnanico E (isómero 1)	-	8.40±0.01
18	13.9	256, 267, 345	447	Luteolina-*C*-hexósido	-	3.20±0.02
19	14.7	274	571	Ácido yunnanico E (isómero 1)	-	4.5±0.2
20	15.0	235, 277	539	Ácido yunnanico D / isómero	-	3.9±0.3
21	15.2	268, 336	431	Apigenina-*C*-hexósido	-	5.9±0.6
22	15.6	285, 315	555	Ácido salvianólico K	-	D
23	15.8	255, 350	463	Quercetina-*O*-hexósido	14.6±0.3	-
			593	Luteolina rutinósido	D	-
24	16.1	255, 266, 345	461	Luteolina-7-*O*-glucurónido	-	15.8±0.02
25	16.5	274	571	Ácido yunnanico E (isómero 2)	-	1.00±0.08
26	17.6	268, 336	575	Apigenina hexóxido 3-hidroxi-3-metilglutaroil	-	6.30±0.02
27	17.9	283	719	Ácido sagerinico (isómero1)	D	2.10±0.08
28	18.1	269, 329	431	Apigenina-*O*-hexósido	-	16.7±0.05
29	18.3	238, 341	607	Crioseriol-*O*-rutinósido	D	-
30	18.4	267, 337	445	Apigenina-*O*-glucurónido	-	2.2±0.01
31	18.6	270, 291, 326	717	Ácido salvianólico B (isómero 1)	-	D
32	18.7	284, 330	609	Hesperidina	41.3±2.2	-
33	19.0	218, 290, 328	359	Ácido rosmarínico	56.8±0.6	17.8±0.1
34	19.5	285, 305	537	Ácido rosmarinico cafeico (isómero 1)	-	1.0±0.04
35	19.8	278	719	Ácido sagerinico (isómero 2)	-	2.2±0.02
36	20.2	290, 333	537	Ácido rosmarinico cafeico/ Ácido salvianólico I (isómero 2)	D	-
37	20.6	267, 336	517	Apigenina malonil hexóxido	-	2.50±0.03

38	21.5	287, 320	373	Rosmarinato de metilo	-	0.60±0.02
39	21.8	290	491	Derivado del ácido cumárico	0.50±0.01	-
40	22.4	281, 330sh	717	Ácido salvianólico B (isómero 2)	6.6±0.4	-
41	23.7	199, 229, 287	361	Derivado de sageone	174.1±14.1	-
42	24.3	275, 333	313	Cirsimaritin	25.9±0.6	-
43	25.0	207, 237, 285	345	Rosmanol	192.4±17.1	-
44	25.2	204, 232, 286	347	Ácido hidroxicarnosico	69.7±11.2	-
45	25.7	286	329	Carnosol	17.3±0.7	-
46	26.3	262	331	Ácido carnósico	14.3±0.7	-
47	26.4	277	301	Tetrahidrohidroxi rosmariquinona	17.4±0.2	-
				Total	643.3±18.9	102.1±0.7

* Valores expresados en mg/g de extracto; D: Detectado; Fr. Fracción; TR: tiempo de retención

Esto estudio sugiere que la alta bioactividad de las decocciones de *S. apiana* se asocia con su abundancia en componentes fenólicos, como los flavonoides y los derivados terpenicos, como se había descrito anteriormente en diferentes especies de *Salvia* [3,30,31]. Algunos de estos compuestos ejercen varias propiedades biológicas, incluida la actividad inhibidora de la lipasa [32], antioxidante, antimicrobiana, antiinflamatoria y antitumoral [30,31,33].

4. Conclusiones

El presente estudio demostró que las decocciones de *S. apiana* y *S. farinacea* var. *victoria blue* tienen propiedades antioxidantes, antiinflamatorias, citotóxicas y antibacterianas, que son fuertemente prometedores en la primera planta. Así, *S. apiana* mostró capacidad para contrarrestar eventos clave en el estrés oxidativo, y suprimió la actividad de células inflamatorias. Además, las acciones de esta decocción fueron selectiva para las celulas tumorales, exhibiendo capacidad citotóxica en líneas celulares de cáncer con bajo efecto citotóxico en células normales. Por otra parte, también ejerció un potencial inhibitorio y letal contra cinco especies bacterianas. Posiblemente, el gran potencial bioactivo de esta decocción puede estar parcialmente asociado con sus altos niveles en compuestos fenólicos (643.3 μg/mg), particularmente en compuestos terpenoides. Aunque también con potencial interés, la bioactividad de *S. farinacea* var. *victoria blue* fue en general significativamente más baja que la de *S. apiana*, y también contenía niveles modestos de compuestos fenólicos (102.1 μg/mg). La excelente actividad *in vitro* antioxidante de la especie de *S. apiana*, junto con sus buenos efectos antiinflamatorios, de citotoxicidad y antibacterianos, la hace especialmente interesante en la aplicación en alimentos y en la industria farmacéutica.

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1 *Article*

2 Health-benefits and phytochemical profile of *Salvia apiana* and 3 *Salvia farinacea* var. *victoria blue* decoctions

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14 Received: date; Accepted: date; Published: date
15

16 **Abstract:** *Salvia apiana* and *Salvia farinacea* var. *victoria blue* decoctions were screened for diverse
17 bioactivities, including the ability to counteract oxidative and inflammatory events, as well as to act
18 as cytotoxic and antimicrobial agents. Regardless both extracts showed good activities, that of *S.*
19 *apiana* origin was particularly effective regarding the ability to prevent lipid peroxidation and to
20 prevent nitric oxide (NO•) production in LPS-activated RAW 264.7 macrophages (EC_{50}=50 μg/mL).
21 Moreover, it displayed high cytotoxic capacity against hepatocellular carcinoma HepG2, cervical
22 carcinoma HeLa and breast carcinoma cells MCF-7 but comparatively low effects in porcine liver
23 primary cells, which highlights its selectivity (GI_{50}= 41-60 μg/mL *vs* 362 μg/mL, respectively). As well,
24 it exhibited inhibitory and lethal potential against a panel of Gram-positive and Gram-negative
25 bacteria. Possibly, the bioactive properties of the two *Salvia* extracts are partially associated to their
26 phenolic components, and in the particular case of *S. apiana*, to its richness in phenolic terpenes,
27 particularly in rosmanol, hydroxycarnosic acid and a derivative of sageone, which were found in the
28 extract.

29 **Keywords:** *Sage*, bioactivity, antioxidant, anti-inflammatory, cytotoxicity, antibacterial, phenolic
30 compounds, terpenes.
31

32 1. Introduction

33 In normal physiological conditions, cells maintain a redox homeostasis, i.e., the balance between
34 reactive species formation and their elimination. Still, when equilibrium is disrupted, oxidative stress
35 is settled and the overproduction of reactive species causes several mitochondrial and cellular
36 damages, namely in lipids, proteins, DNA and other macromolecules. Ultimately, these events are
37 known to be associated with aging and the onset and/or development of distinct diseases, including
38 cancer [1,2]. In this context, diet supplementation with plant-based products rich in phytochemicals
39 are believed to counteract oxidative-related events, thus contributing to human health [1,3,4].

40 *Salvia* is the largest genus in the Lamiaceae family plants, with over 900 species [5], being claimed
41 for their richness in essential oils and/or phenolic compounds [6–9], a fact that is closely associated
42 to their usage in traditional medicine and potential applications in distinct industries, including food,

pharmaceutical and cosmetics [4,10]. *Salvia farinacea* var. *victoria blue* and *Salvia apiana* are two edible *Salvia* plants native to southwestern regions of the USA and Mexico. The first is a perennial shrub typically growing 45-90 cm, whose gray-green leaves are drooping, irregularly-serrate, ovate-lanceolate and it contains a compact multi-branched rich violet-blue flowers spikes, resembling lavender [11–13]. In turn, *S. apiana*, also known as white sage, is a branched shrub growing up to 1.5 m, with silvery white petiolate leaves highly aromatic, and white to pale lavender flowers [14,15].

Although low exploited, *S. apiana* has been previously studied regarding its essential oils composition, which are known to contain high amounts of terpenes and terpene derivatives, particularly β-pinene, α-pinene, borneol, 1,8-cineole, campholenic acid and β-caryophyllene [13,16]. Moreover, phytochemical analysis of hydroethanolic or acetone extracts of this species have allowed the identification of distinct diterpenes and triterpenes, and the flavonoids cirsimaritin and salvigenin [17,18]. Still, as far as we know, the biological properties of polar extracts from *S. apiana* aerial parts were limited to screen of the cannabinoid or opioid receptors activity of an ethanolic extract [17] and the potential cytotoxicity against leukemia cell lines of a methanolic extract [19]. As regard to *S. farinacea* var. *victoria blue*, both chemical and biological properties of polar extracts remain unexplored. Hence, the present study aimed to investigate potential biological effects of *S. apiana* and *S. farinacea* var. *victoria blue*, having in mind the traditional usage of *Salvia* species, which are used for centuries in form of infusions and decoctions. The antioxidant, anti-inflammatory, cytotoxicity and antibacterial potencies of decoctions will be associated with their specific phenolic components.

2. Materials and Methods

2.1. Chemicals

Rosmarinic acid and the 7-*O*-glucoside derivatives of apigenin, luteolin, eriodyctiol, quinic acid, caffeic acid, 5-*O*-caffeoylquinic acid, 4-*O*-hydroxybenzoic acid, salvianolic acid B, caffeic acid, ferulic acid and 4-*O*-coumaric acid were obtained from Extrasynthese (Genay Cedex, France). Trolox, sulforhodamine B (SRB), DPPH (2,2-diphenyl-2-picrylhydrazyl) radical, acetic acid, ellipticine, trypan blue, trichloroacetic acid (TCA), Tris, lipopolysaccharide (LPS), carnosol, nisin, ascorbic acid and butylated hydroxyanisole (BHA) were obtained from Sigma Chemical Co (St Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). The Griess reagent system was purchased from Promega Corporation (Madison, WI, USA). Mueller-Hinton agar was from VWR (Prolabo Chemicals, USA); formic acid and ethanol was from Panreac (Barcelona, Spain). *n*-Hexane, methanol and acetonitrile were purchased from Lab-Scan (Lisbon, Portugal). Purified water was obtained from a Direct-Q® water purification system (Merck Life Science, Germany).

2.2. Plant material

The *S. apiana* and *S. farinacea* var. *victoria blue* species were collected from the fields of Coimbra College of Agriculture, Portugal, GPS coordinates 40.211439, -8.451251. After collection, its aerial parts were dried in a ventilated incubator at 35ºC for 3 days and kept in dark room until use.

2.3 Preparation of extracts

82 Extraction of the phenolic compounds was performed by decoction, following a method
83 previously described [20], with slight changes. Briefly, the aerial parts (flowers, leaves and stems) of
84 sage plants were boiled for 15 minutes (5 g of plant in 100 mL of water), filtrated and concentrated to
85 about half of the volume in a rotary evaporator, to reduce the amount of water, followed by defatting
86 with *n*-hexane (1:1 *v/v*). Traces of *n*-hexane in the aqueous phase were eliminated by rotary
87 evaporation before freeze-drying. The resulting extract was kept under vacuum in a desiccator in the
88 dark and a stock water solution (10 mg/mL) was prepared just before the analysis.

89 *2.4. Identification and quantification of phenolic compounds*

90 UHPLC-DAD-ESI/MS^n analyses of phenolic profiles from the two decoctions (5 mg/mL) were
91 carried out on an Ultimate 3000 (Dionex Co., San Jose, CA, USA) apparatus equipped with an
92 ultimate 3000 Diode Array Detector (Dionex Co., San Jose, CA, USA) and coupled to a Thermo LTQ
93 XL mass spectrometer (Thermo Scientific, San Jose, CA, USA), an ion trap MS equipped with an
94 electrospray ionization (ESI) source, following a method previous described [21]. Gradient elution
95 was carried out with a mixture of 0.1% (*v/v*) of formic acid in water (solvent A) and acetonitrile
96 (solvent B). The solvent gradient used consisted of a series of linear gradients starting from 5% of
97 solvent B and increasing to 23% at 14.8 min, to 35% at 18 min, and to 100% at 21 min over three
98 minutes, followed by a return to the initial conditions. The mass spectrometer used was a Thermo
99 Xcalibur Qual Browser (Thermo Scientific, USA) and the operations were carried out using the
100 conditions previously described [22]. Quantification was performed by the external standard method
101 using the calibration curves of structurally-related standard compounds. Considering the nature of
102 the phenolic compounds, their quantification was performed at 280, 320 or 340 nm, also considering
103 the detection and quantification limits (LOD and LOQ, respectively). LOD and LOQ were determined
104 from the parameters of the calibration curves, being defined as 3.3 and 10 times the value of the
105 regression error divided by the slope, respectively [21].

106 *2.5. Antioxidant activity*

107 2.5.1. DPPH• scavenging test

108 The extracts capacity for scavenging DPPH• was evaluated following the procedure previously
109 described by Catarino et al. [23]. Ascorbic acid was used as positive control and the results were
110 expressed as EC_{50} values (sample concentration providing 50% of antioxidant activity).

111 2.5.2. Ferric reducing power assay

112 The ability of the two sage extracts to reduce iron (III) to iron (II) was carried out according to a
113 procedure described before [23], on which the antioxidant compounds from the extracts form a
114 colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, measurable at
115 700 nm. BHA (2,6-di-*tert*-butyl-4-methylphenol) was used as positive control and the results were
116 expressed as EC_{50} values.

117 2.5.3. Thiobarbituric acid reactive substances (TBARS)

118 The decrease in thiobarbituric acid reactive substances (TBARS) was evaluated by the inhibition
119 of lipid peroxidation in porcine (*Sus scrofa*) brain homogenates, according to a procedure described
120 by Martins et al. [24]. The color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA)

was measured as its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following equation: [(A−B) / A] × 100%, where A and B were the absorbance of the control and the sample solutions, respectively. Trolox was used as positive control and the results were expressed as EC_{50} values.

2.5.4. β-Carotene bleaching assay

β-carotene linoleate general assay was performed as described by Barros et al. [25]. Trolox was used as positive control and the results were expressed as EC_{50} values.

2.6. Anti-inflammatory activity

The extracts ability in scavenging the NO radical was evaluated in the mouse macrophage-like cell line RAW 264.7 following the general procedure previously described [26]. Cells were treated under 5% CO_2 in humidified air, using DMEM culture medium enriched with 10% heat inactivated fetal bovine serum, glutamine and antibiotics at 37 ºC. For the tests, cells were seeded in 96-well plates (150,000 cells/well) and allowed do attach to the plate overnight. Then, these were treated with extract solutions (concentration of 25 and 100 μg/mL, for each extract) for 1 h, followed by the stimulation with LPS (1 μg/mL) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in nitric oxide ($NO^{\bullet}$) basal levels. Dexamethasone (50 μM) was used as a positive control while negative controls had no added LPS. The NO levels produced were determined by the Griess reaction, used for measuring the nitrite accumulation in the culture supernatant on macrophage cell line RAW 264.7 [26]. The anti-inflammatory activity of each extract was determined by calculating EC_{50} values (μg/mL), which corresponds to the sample concentration that provides 50% inhibition of NO production.

2.7. Cytotoxic effect in four human tumor cell lines

The cytotoxic effect of *S. apiana* and *S. farinacea* var. *victoria blue* extracts towards four human tumor cell lines were carried out by the sulforhodamine B (SRB) assay, using the conditions established by Souza et al. [26]. The tumor cell lines MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma), were maintained in enriched medium, at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5 × 103 cells/well for MCF-7 and NCI-H460 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates. The cytotoxicity results were expressed as GI_{50} values (μg/mL), corresponding to sample concentration that inhibited 50% of the cell growth. Ellipticine was used as positive control.

2.8. Cytotoxic effect in non-tumor liver cells

The cytotoxicity of the sage extracts were tested by hepatotoxicity assay, in a primary non-tumor cell culture obtained from porcine liver (PLP2) following the described procedures [26]. For the tests, cells were seeded (at 1.0×10^4 cells/well) in enriched medium. Results were expressed as GI_{50} values (μg/mL) and ellipticine was used as a positive control.

2.9. Antimicrobial activity

The antibacterial potential of the *S. apiana* and *S. farinacea* var. *victoria blue* species was evaluated against five bacterial strains, including Gram-positive bacteria (*Staphylococcus epidermidis* NCTC

160 11047 and *Staphylococcus aureus* NCTC 6571) and Gram-negative bacteria (*Salmonella typhimurium*
161 NCTC 12023, *Escherichia coli* NCTC 9001, and *Pseudomonas aeruginosa* NCTC 10662) from the National
162 Collection of Type Cultures, operated by Public Health England. All strains were cultured in Mueller-
163 Hinton agar and incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) and
164 minimum bactericidal concentration (MBC) of aqueous solutions of both *Salvia* species were
165 determined by the broth microdilution method using an adapted method previous described by
166 Afonso et al [21]. MIC is defined as the lowest concentration at which visible growth is inhibit, while
167 MBC is the lowest concentration of the tested substance which has a bactericidal effect. Briefly,
168 bacterial suspensions were prepared by direct colony suspensions and a final inoculum of 1.5×10^5
169 CFU/mL was required for final suspensions that was diluted in a 1:100 ratio in Mueller-Hinton broth.
170 One hundred microliters of this medium was dispensed into wells of 96-well micro titer plates and
171 *S. apiana* and *S. farinacea* var. *victoria blue* decoctions were added and serially diluted four times across
172 the plate. One hundred microliters of bacteria suspension was finally added to each well and the
173 plates were incubated at 37 °C for 24 h. The assay for each pathogen was repeated three times [27].
174 MBC values are determined by sub-culturing from each negative well onto Mueller-Hinton agar and
175 confirmation the lowest concentration with no visible growth [28]. Nisin was used as the positive
176 control.

177 *2.10. Statistical analysis*

178 The results were analyzed using GraphPad Prism 6 (GraphPad Software, CA, USA). Data were
179 expressed as mean ± S.D. of 3-4 independent experiments performed at least in triplicate. One-way
180 analysis of variance (ANOVA) followed by Tukey´s test was used to detect any significant differences
181 among different means. The *p*-value less than 0.05 were assumed as significant difference.

182 3. Results and discussion

183 3.1. Antioxidant activity

184 Many plants, including *Salvia* species, have been evaluated for their claimed antioxidant
185 capacities and/or ability to ameliorate oxidative-stress related disorders, being these properties
186 commonly associated to the richness in phenolic compounds [4, 27].
187

188 **Table 1 –** Antioxidant activity (EC_{50}, μg/mL) of *S. apiana* and *S. farinacea* var. *victoria blue* decoctions

Assay	*S. apiana*	*S. farinacea* var. *victoria blue*	Standard		
			AA	BHA	Trolox
DPPH•	$13.3±1.1^a$	$17.4±5.5^a$	$6.7±0.7^b$		
Ferric Reducing Power	$55.0±5.6^a$	$59.9±3.6^a$		$16.1±2.0^b$	
TBARS	$2.79±0.1^a$	$42.2±0.6^b$			$23.0±1.0^c$
β-carotene bleaching inhibition	$41.2±1.6^a$	$153.5±2.2^b$			$41.7±0.3^a$

189 AA - Ascorbic acid; BHA - butylated hydroxyanisole; DPPH - 2,2-diphenyl-1-picrylhydrazyl; TBARS -
190 thiobarbituric acid reactive substances. Means followed by the same letters in rows do not differ by Tukey's test
191 ($p < 0.05$).

192 The antioxidant activity of *Salvia* decoctions was evaluated by four generalized methods, namely
193 DPPH•, ferric reducing power assay, bleaching of β-carotene and TBARS. Globally, *S. apiana*
194 decoction presented superior antioxidant capacity than that of S. *farinacea* var. *victoria blue*, a trend

195 that was particularly evident with respect to lipid peroxidation events (Table 1), with EC_{50} values
196 obtained of about one-eight or the same magnitude of the commercial standard, in the TBARS and β-
197 carotene bleaching assays, respectively. Regardless of being less active, note that *S. farinacea* var.
198 *victoria blue* extract may also be considered as a promising antioxidant agent, since EC_{50} values in all
199 tests were only 1.8-3.7 fold those of the standard compounds.

200 As far as we know, the antioxidant capacities of *S. apiana* and *S. farinacea* var. *victoria blue* polar
201 extracts have not been exploited before. However, the promising abilities herein reported for
202 decoctions agree and/or, in particular in the case of *S. apiana*, have exceeded those previously
203 described for sage plants [7,6,28,29].

204 *3.2. Anti-inflammatory activity*

205 Nitric oxide produced in high concentrations by macrophages has been reported to be involved
206 in inflammation and compounds targeting its production are considered as good candidates for
207 attenuating inflammatory-related diseases [30]. In our study, the potency of *S. apiana* and *S. farinacea*
208 var. *victoria blue* extracts to counteract NO• production was evaluated in an LPS-activated RAW 264.7
209 macrophage model. As shown in Table 2, both extracts were effective, and particularly that of *S.*
210 *apiana* was quite promising as its potency corresponded to about one third of the drug
211 dexamethasone (EC_{50}=49.9±2.5 and 16.0±1.0, respectively). Moreover, their activity seems to be
212 superior to that described by Ravipati et al. [31] and by Jang et al [32] for commercial aqueous extracts
213 of *Salvia miltiorrhiza* (EC_{50}=200 μg/mL) and for the 80% ethanolic extracts of aerial parts and of roots
214 of *Salvia plebeia* (EC_{50} between 500-1000 and superior to 1000 μg/mL), albeit that the absence and/or
215 the use of a different reference compound by the authors hamper more solid conclusions. In addition,
216 no relationship was made by the authors between the anti-inflammatory effects and the possible
217 compounds involved.

218 **Table 2 –** Anti-inflammatory (EC_{50}, μg/mL) and cytotoxicity properties (GI_{50}, μg/mL) of *S. apiana*
219 and *S. farinacea* var. *victoria blue* decoctions.

	S. apiana	***S. farinacea* var. *victoria blue***	**Standard**
Anti-inflammatory activity			*Dexamethasone*
NO• production	49.9±2.5[a]	80.8±0.4[b]	16.0±1.0[c]
Cytotoxicity to tumor cell lines			*Ellipticine*
HepG2 (hepatocellular carcinoma)	40.9±3.3[a]	87.4±5.4[b]	1.0±0.2[c]
HeLa (cervical carcinoma)	57.3±5.1[a]	77.8±3.5[b]	2.0±0.1[c]
MCF-7 (breast carcinoma)	60.2±4.2[a]	59.8±0.1[a]	1.0±0.04[b]
NCI-H460 (non-small cell lung cancer)	245.7±6.3[a]	279.5±10.1[b]	1.0±0.1[c]
Cytotoxicity to non-tumor cells			
PLP2 growth inhibition	361.7±5.3[a]	335.4±8.0[b]	3.0±1.0[c]

220 Means followed by the same letters in rows do not differ by Tukey's test ($p < 0.05$).

221

222 *3.3. Cytotoxic activity*

223 The effect of the two *Salvia* decoctions on the growth of tumor and non-tumor cell lines was
224 evaluated by the sulforhodamine B (SRB) assay. In general, both *Salvia* extracts showed promising
225 cytotoxic effects against tumoral cell lines, particularly hepatocellular carcinoma HepG2, cervical
226 carcinoma HeLa and breast carcinoma cells MCF-7. Again, between the two extracts, *S. apiana*
227 exhibited more cytotoxicity than that of *S. farinacea* var. *victoria blue*, a fact that was clear in HepG2
228 cells (GI_{50} of 40.9±3.3 *vs* 87.4±5.4 μg/mL, respectively) and HeLa (GI_{50} of 57.3±5.1 *vs* 77.8±3.5 μg/mL,
229 respectively). Note that these data seems to reinforce the results of Saeed et al. [19], who reported a
230 high cytotoxic effect of *S. apiana* methanolic extracts against both sensitive and multidrug-resistant
231 leukemia cells (GI_{50} values of 7.17±0.67 and 9.91±0.8 μg/mL, respectively), as assessed by the resazurin
232 reduction assay. As well, although our results cannot be directly compared to those reported
233 previously for other plants (due to the absence or use of different standards or methodologies), they
234 generally reinforce the theory that polar extracts of sage plants may act as cytotoxic agents. In fact,
235 other authors have previously highlighted cytotoxic effects for such extracts, e.g *Salvia eremophila*
236 methanolic or hydromethanolic extracts, which were effective in breast cancer MCF-7 cell lines (GI_{50}
237 values of 47.7 and 75.2 μg/ml, respectively), with 7 to 11 - fold less potency of the drug cisplatin [33].
238 Using the MTS assay, Jiang et al. [34] also highlighted a high cytotoxicity capacity of ethanolic and
239 acetone extracts from *S. miltiorrhiza* roots against HepG2 cell lines (GI_{50}=17.3 and 83.2 μg/ml,
240 respectively), as well as of *Salvia officinalis* roots and leaves (GI_{50}=19.6-43.8 μg/ml and 64.4-90.0 μg/ml,
241 respectively).

242 Moreover, our results also indicated that the anti-proliferative activity of *S. apiana* and *S. farinacea*
243 var. *victoria blue* decoctions were tumor-selective, as reflected by comparatively low GI_{50} values in the
244 cancer cell lines compared to those of PLP2 cells (GI_{50}=361.7 and 335.4 μg/mL, respectively). This
245 ability to discriminate tumor cells is essential to minimize cytotoxic effects in normal cells, and has
246 also been described for other *Salvia* polar extracts, including *S. officinalis,* which regardless being
247 cytotoxic to MCF-7 cancer cells (GI_{50}=142.4 μg/mL), showed no significant effects against normal
248 human umbilical vein endothelial cell cells (IC_{50} values above 600 μg/mL) [19].

249 *3.4. Antibacterial activity*

250 The antibacterial potential of the two *Salvia* decoctions towards the Gram-positive bacteria *S.*
251 *aureus* and *S. epidermidis* and the Gram-negative bacteria *S. typhimurium*, *E. coli* and *P. aeruginosa* are
252 summarized in Table 3. In general, *S. apiana* had promising antibacterial properties, with inhibitory
253 and lethal capacity at concentrations equal or below to 0.69 mg/mL (for *S. aureus* and *S. epidermidis*)
254 and of 2.75 mg/mL (for the *S. typhimurium*, *E. coli* and *P. aeruginosa)*, which corresponded to only 3-5
255 times less that of the drug nisin. This contrasted with *S. farinacea* var. *victoria blue* decoction, that only
256 showed relevant antibacterial effect towards the Gram-positive bacteria *S. aureus* (MIC and MBC of
257 1.06 and 2.12 mg/mL, respectively).

258 As far as we know, the antibacterial effects of *S. apiana* and *S. farinacea* var. *victoria blue* polar
259 extracts have not been previously exploited. Still Córdova-Guerrero et al. [35] has remarked the
260 antimicrobial activity of a *S. apiana* hexane extract against Gram-positive bacteria, using the agar disc
261 diffusion assay, for which they obtained an inhibition hale of *S. aureus* in the range of 10 to 24 mm at
262 0.34 – 2.7 μg/μL. Unfortunately, as the authors did not present the MIC value, a direct comparison to
263 our results is not possible. Nonetheless, other authors have previously described promising

264 antimicrobial effects of polar extracts of sage species. E.g., Ibrahim et al [36] described a powerful
265 activity of *Salvia bicolor* methanolic extract against a distinct Gram-positive bacteria, especially on *S.*
266 *aureus* and *S. epidermidis* (MIC values of 0.2 and 0.35 mg/mL, respectively) and Gram-negative
267 bacteria (MIC values in the range 0.4 to 1 mg/mL) [36], a fact that the authors associated with its
268 richness in phenolic compounds, which were mostly gallic, *p*-coumaric and protocatechuic acids, and
269 flavonoids (e.g. luteolin-7-*O*-glucoside). Moreover, when screening the antimicrobial potential of
270 hydromethanolic extracts of different *Salvia* species through the nutrient-broth microdilution assay,
271 Firuzi et al. [33] reinforced that *S. eremophila, Salvia limbata, Salvia santolinifolia* and *Salvia sclarea*
272 extracts were able to inhibit the growth of a panel of six microorganisms, with MIC values in the
273 range of 0.31-5 mg/mL.

274 **Table 3 –** MIC (mg/mL) and MBC (mg/mL) of extracts of *S. apiana* and *S. farinacea* var. *victoria blue*
275 and nisin (mg/mL) against selected test bacteria.

	S. apiana		***S. farinacea* var. *victoria blue***		**Nisin**	
Bacteria	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**
Gram-positive						
S. epidermidis	0.69	0.34	8.50	8.50	<0.63	<0.63
S. aureus	0.69	0.69	2.12	1.06	<0.63	<0.63
Gram-negative						
S. typhimurium	2.75	2.75	>8.5	>8.5	0.5	0.5
E. coli	2.75	2.75	8.5	8.5	1.0	0.5
P. aeruginosa	2.75	2.75	>8.5	>8.5	1.0	1.0

276 MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

277 *3.5. Characterization of the extracts*

278 Not surprisingly, the levels of phenolic compounds in *Salvia* decoctions were significantly
279 different, with *S. apiana* being about six times richer than *S. farinacea* var. *victoria* (Table 4). Moreover,
280 regardless of being rich in rosmarinic acid (56.8 and 17.8 mg/g extract, respectively) as in general for
281 polar extracts from *Salvia* plants, the two extracts differed with regard to their phenolic constituents,
282 as well as to those previously reported for *Salvia elegans*, *Salvia greggii* and *S. officinalis* decoctions [37].
283 Indeed, *S. apiana* decoction was characterized by its richness in terpenes derivatives (peaks 41 and
284 43-47, in Figure 1 and Table 4), which were mainly represented by rosmanol ($[M-H]^-$ at *m/z* 345⟶
285 301, 271, 283), a derivative of sageone ($[M-H]^-$ at *m/z* 361) and hydroxycarnosic acid ($[M-H]^-$ at *m/z*
286 347⟶ 303, 273), with estimated levels of 192.4±17.1, 174.1±14.1 and 69.7±11.2 mg/g extract,
287 respectively, and other less abundant ones, including carnosol, carnosic acid and
288 tetrahydrohydroxyrosmariquinone (peaks 45, 46 and 47, respectively), in addition to some flavonoids
289 (hesperidin, quercetin-*O*-hexoside and cirsimaritin). These results strengthen those previously
290 reported [17,18,38], allowing to conclude that polar extracts from *S. apiana* are particularly rich in
291 phenolic terpenes. Note that, among the compounds herein detected in the decoction of *S. apiana*,
292 rosmanol and hydroxycarnosic acid has been previously described in the acetone extracts [18], while
293 carnosol, sageone and carnosic acid were reported in aqueous ethanolic and/or methanolic extracts

294 from the same plant [17,38]. In turn, the diterpenes rosmadial, 16-hydroxycarnosol, 16-
295 hydroxyrosmanol, salvicanol and the triterpenes oleanolic acid, uvaol and ursolic acid were found in
296 *S. apiana* polar extracts by other authors [17,18].
297 Although less rich in phenolic compounds, in addition to rosmarinic acid, *S. farinacea* var. *victoria*
298 *blue* decoction presented moderate quantities of common flavones to the *Salvia* plants, namely
299 apigenin-*O*-hexoside (peak 28, 16.7 mg/g extract) and luteolin-*O*-glucuronide (peak 24, 15.8 mg/g
300 extract) [39–41].

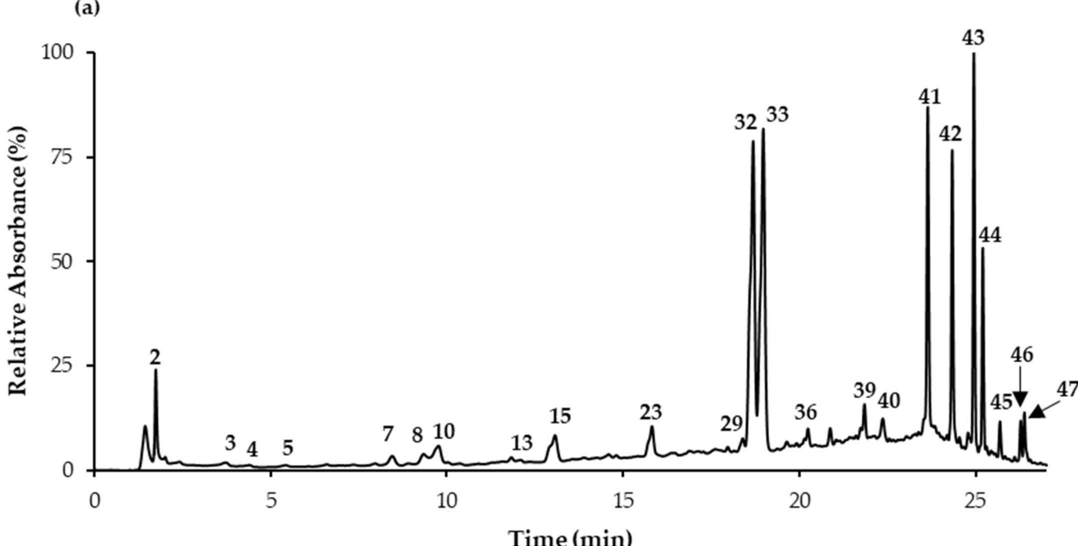
301 **Table 4 –** Identification and quantification of the compounds identified in *S. apiana* and *S. farinacea*
302 var. *victoria blue* decoctions determined by UHPLC-DAD-ESI-MS^n.

NF	Rt	UV max	$[M-H]^-$	MS^2 Main fragments ESI- MS^n (intensity)	Probable Compound	*S. apiana**	*S. farinacea* var. *victoria blue**
1	1.5	275	149	103, 87, 131, 59	DimethylBA	-	5.9±0.1
2	1.7	205	191	111, 173	Quinic Ac	5.0±0.3	0.4±0.01
3	3.6	280	197	179, 73, 153	Danshensu	D	D
4	4.4	261, 289	153	109	Protoc Ac	D	-
5	5.1	290sh, 324	353	191, 179, 135	*cis* 3-*O*-CQA	D	-
6	5.4	294sh, 322	353	191, 179, 135	*trans* 3-*O*-CQA	-	D
7	7.9	309	337	163	Coum Quinic Ac	D	0.3±0.03
8	8.3	313	295	163	*p*-Coum Ac Pent	0.4±0.04	-
9	8.8	290sh, 325	353	191	*trans* 5-*O*-CQA	-	0.6±0.03
10	9.5	290sh, 325	353	173, 179, 191	4-*O*-CQA	5.5±0.1	-
11	9.7	290sh, 323	179	135	Caff Ac	-	0.8±0.0
12	9.8	314	325	265, 235, 163	Coum Hex	-	0.3±0.0
13	11.8	311	337	191, 163	Coum Quinic Ac	D	0.2±0.0
14	12.8	287sh, 324	367	173, 191	Fer Quinic Ac	-	D
15	13.0	309	225	207, 181, 165, 163	Coum Ac Der	1.8±0.1	-
16	13.1	291sh, 311	637	351, 285, 193	Ferulic Ac Der	-	0.5±0.0
17	13.5	274	571	527, 483, 439, 373	YA E (isom1)	-	8.4±0.01
18	13.9	256, 267sh, 345	447	327, 357	Lut-*C*-Hex	-	3.2±0.02
19	14.7	274	571	527, 509, 553, 483, 285	YA E (isom1)	-	4.5±0.2
20	15.0	235, 277	539	297, 359, 377, 279, 315	YA D/isomer	-	3.9±0.3
21	15.2	268, 336	431	311, 341, 269	Api-*C*-Hex	-	5.9±0.6
22	15.6	285, 315	555	409, 391, 537, 511, 365	SA K	-	D
23	15.8	255, 350	463	301	Querc-*O*-Hex	14.6±0.3	-
			593	285	Lut Rut	D	-
24	16.1	255, 266, 345	461	285	Lut-7-*O*-GlcA	-	15.8±0.02

25	16.5	274	571	527, 409	YA E (isom2)	-	1.0±0.08
26	17.6	268, 336	575	431, 341, 311, 513, 413	Api Hex HMG	-	6.3±0.02
27	17.9	283	719	359, 539, 521, 341	Sag Ac (isom1)	D	2.1±0.08
28	18.1	269, 329	431	269	Api-*O*-Hex	-	16.7±0.05
29	18.3	238, 341	607	299, 284	Chrys-*O*-Rut	D	-
30	18.4	267, 337	445	269, 175	Api-*O*-GlcA	-	2.2±0.01
31	18.6	270, 291, 326	717	555, 519, 475, 357	SA B (isom1)	-	D
32	18.7	284, 330sh	609	301	Hesperidin	41.3±2.2	-
33	19.0	290sh, 328	359	161, 179, 197, 223	RA	56.8±0.6	17.8±0.1
34	19.5	285sh, 305	537	493, 295	CaffRA (isom1)	-	1.0±0.04
35	19.8	278	719	521, 341, 359	Sag Ac (isom2)	-	2.2±0.02
36	20.2	290sh, 333	537	493, 359, 375	CaffRA/ SA I (isom2)	D	-
37	20.6	267, 336	517	269, 473	Api malonyl Hex	-	2.5±0.03
38	21.5	287sh, 320	373	179, 161, 135, 197, 355, 329	Methyl Rosmarinate	-	0.6±0.02
39	21.8	290	491	163, 329, 119	Coumaric Ac Der	0.5±0.01	-
40	22.4	281, 330sh	717	537, 357	SA B (isom2)	6.6±0.4	-
41	23.7	199, 229, 287	361	299, 269, 281, 213, 343	Sageone Der	174.1±14.1	-
42	24.3	275, 333	313	298, 283, 269	Cirsimaritin	25.9±0.6	-
43	25.0	207, 237sh, 285	345	301, 271, 283	Rosmanol	192.4±17.1	-
44	25.2	286	347	303, 273	Hydroxycarnosic Ac	69.7±11.2	-
45	25.7	286	329	285	Carnosol	17.3±0.7	-
46	26.3	262	331	287	Carnosic Ac	14.3±0.7	-
47	26.4	277	301	271, 283	Tetrahydrohydroxyrosmariquinone	17.4±0.2	-
					Total	643.3±18.9	102.1±0.7

303 *Values expressed as mg/g of extract; NF- Number of peak represented in Figure 1; D- Detected; Ac- acid; Api-
304 Apigenin; BA- Benzoic acid; CaffAc- Caffeic acid; Caff- Caffeoyl; CQA- Caffeoylquinic acid ; Chrys- Chrysoeriol;
305 Coum- Coumaroyl Der- Derivative; Fer- Feruloyl; Glc- Glucoside; GlcA- Glucuronide; Hex- Hexoside; HMG- 3-
306 hydroxy-3-methylglutaroyl; Hy - hydroxy; Lut- Luteolin; Pent- Pentoside; Protoc- Protocatechuic; Querc-
307 Quercetin; Rut- Rutinoside; RA- Rosmarinic acid; Sag- Sagerinic; SA- Salvianolic acid; Scut- Scutellarein; YA-
308 Yunnaneic acid.

309
310



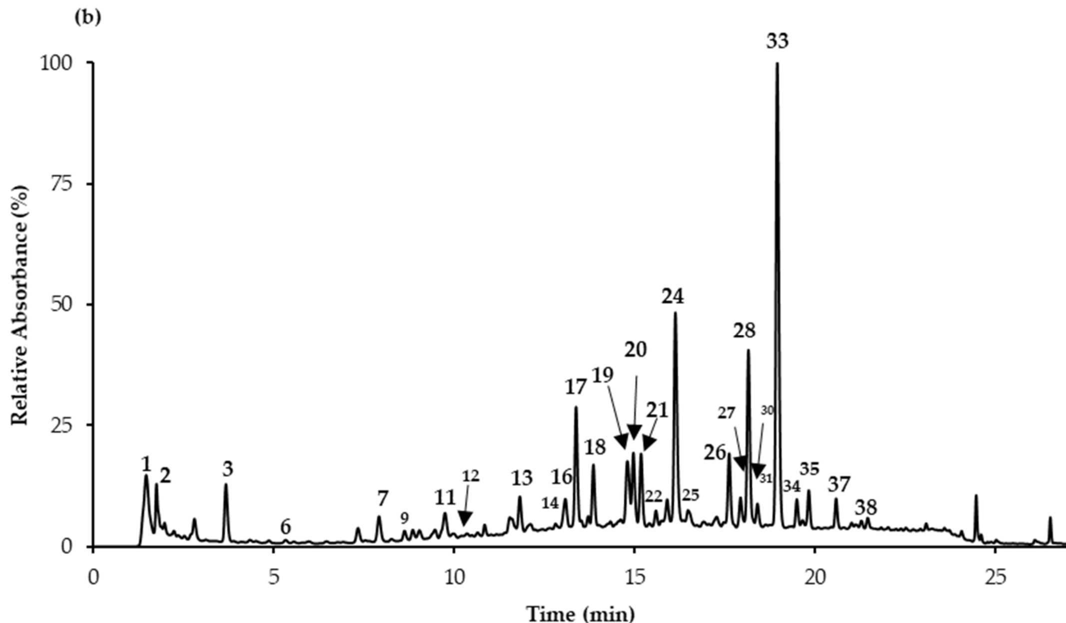


Figure 1. Chromatographic representation of *Salvia* decoctions at 280 nm: (a) *S. apiana*; (b) *S. farinacea* var. *victoria blue*. Numbers in the figure correspond to the UHPLC-DAD-ESI-MS^n peaks described in Table 4.

Thus, our study suggests that the high bioactivity of *S. apiana* decoctions is associated with its fertility in phenolic components, namely flavonoids and terpene derivatives. In fact, the diterpene rosmanol (the most predominant compound in *S. apiana* extract) and its derivatives have previously been associated with the high antioxidant potential of *S. officinalis* [42,43] and the cytotoxic activity of several other *Salvia* plants [8]. In addition, sageone is an abietane diterpenoid previously reported in *S. apiana* species [17] that has already been described to exert antiviral activity [8]. Moreover, others, including carnosol and carnosic acid, are claimed to exert several biological properties, including lipase inhibitory activity [44], antioxidant, antimicrobial, anti-inflammatory and antitumoral [42,43,45]. Naturally, the possible contribution of other components of the extract, including

324 cirsimaritine, which have already been considered relevant in the antibacterial and antioxidant
325 activities of *Salvia* species [9], should not be overlooked [8,43].

326 4. Conclusions

327 The antioxidant, anti-inflammatory, cytotoxic and antibacterial properties of *S. apiana* and *S. farinacea*
328 var. *victoria blue* decoctions were reported in the present work, allowing to conclude that both have
329 potential bioactive effects, which were extremely promising in the first, particularly with regard to
330 counteract key events in oxidative stress, showing EC_{50} values of 2.79 μg/mL and 41.2 μg/mL in
331 TBARS and β-carotene bleaching assays, respectively. Decoction of *S. apiana* had also an effect on the
332 suppression of inflammatory events, since it displayed a third of the potency of the drug
333 dexamethasone on the inhibition of NO• production by LPS-activated RAW 264.7 macrophages. In
334 addition, this decoction was selective for tumors, exhibiting cytotoxic capacity in cancer cell lines
335 with low cytotoxic effect in normal cells. As well, it exerted inhibitory and lethal potential against
336 five bacteria (MIC and MBC equal or below to 0.69 and 2.75 mg/mL for Gram-positive and Gram-
337 negative bacteria, respectively). Possibly, the great bioactive potential of this decoction might be
338 partially associated to its high levels in phenolic compounds (643.3±18.9 μg/mg), particularly in
339 terpene compounds mainly rosmanol, a derivative of sageone and hydroxycarnosic acid. In turn,
340 although promising, the bioactivity of *S. farinacea* was in general significantly lower than that of *S.*
341 *apiana*, and also contained modest levels of phenolic compounds (102.1±0.7 μg/mg) comprises
342 moderate quantities of rosmarinic acid and glycosidic forms of the flavones apigenin and luteolin.
343 The promising biological activities of *S. apiana and S. farinacea* var. *victoria blue* species herein found
344 through *in vitro* methods suggest that decoctions of these species, and particularly that of *S. apiana*,
345 might be useful for the application in the food and pharmaceutical industry, although their health-
346 promoting potencies need to be further proved in vivo and pre-clinical studies.

347 **Author Contributions:** Andrea F. Afonso contribution to investigation, data curation, writing the original draft.
348 Olívia R. Pereira contribution to conceptualization, data curation, supervision, and writing – review and editing.
349 Ângela S. F. Fernandes contribution to investigation (antioxidant assays). Ricardo C. contribution to
350 investigation (cellular assays), writing – review and editing. Artur M. S. Silva contribution to resources and
351 writing – review. Isabel C.F.R. Ferreira contribution to conceptualization, resources and writing – review. Susana
352 M. Cardoso contribution to conceptualization, investigation, supervision, resources and writing – review.

353 **Funding:** Foundation for Science and Technology (FCT), the European Union, the National Strategic Reference
354 Framework (QREN), the European Regional Development Fund (FEDER), and Operational Programme
355 Competitiveness Factors (COMPETE), for funding the Organic Chemistry Research Unit (QOPNA) (FCT
356 UID/QUI/00062/2019) and Mountain Research Center (CIMO) (UID/AGR/00690/2019), through national funds
357 and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement. A. Fernandes and
358 R. Calhelha thank the national funding by FCT, P.I., through the institutional scientific employment program-
359 contract. Project AgroForWealth (CENTRO-01-0145-FEDER-000001), funded by Centro2020, through FEDER
360 and PT2020, financed the research contract of Susana M. Cardoso.

361 **Acknowledgments:** We acknowledge Dr. Filipe Melo from the School of Agriculture of the Polytechnic Institute
362 of Coimbra, for providing the plant material.

363 **Conflicts of Interest:** The authors declare no conflict of interest.

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5. CONCLUSIONS

First conclusion: The *Salvia* and *Thymus* decoctions focused in this study contained relevant amounts of phenolic compounds, in particular of phenolic acids and flavonoids. Those of six thyme origin were mostly rich in caffeic acid derivatives, namely in rosmarinic acid and caffeoyl rosmarinic acids. These compounds were particularly relevant in *T. pulegioides* and *T. fragrantissimus* extracts (rosmarinic acid accounting for about 81 mg/g extract). Moreover, some extracts contained considerable amounts of flavones, namely isomers of luteolin-*O*-glucuronide, particularly in *T. pseudolanuginosus* and *T. pulegioides* (54 and 26 mg/g extract, respectively).

The eight sage decoctions were also rich in rosmarinic acid, being the maximum amount found in *S. africana* (77.0 mg/g extract). Other caffeic acid derivatives were present in abundance in some sage species, namely caffeoylrosmarinic acid and salvianolic acid B (in *S. elegans*) and yunnaneic acid derivatives (in *S. africana*). The majority of *Salvia* species showed also moderate to high percentages of glycosidic flavones, in particular of luteolin-*O*-hexoside and apigenin-*C*-hexoside (in *S. greggii*) and apigenin-*O*-hexoside and luteolin-*O*-glucuronide (in *S. farinacea* var*. victoria blue*), with overall concentration in the range 16 to 26 mg/g extract. In addition, *S. officinalis* and its cultivar *S. officinalis 'Icterina'* were characterized by high abundancy of the *O*-hexuronic form of apigenin (48 and 34 mg/g extract respectively) and scutellarein. *S. mexicana* decoction was the poorest in phenolic compounds, while *S. apiana* was particularly rich in phenolic terpenes, mainly in rosmanol, a derivative of sageone and in hydroxycarnosic acid (192, 174 and 70 mg/g extract, respectively).

Second conclusion: The six *Thymus* decoctions possessed high antioxidant activity as determined by the two chemical assays, DPPH$^{\bullet}$ scavenging and ferric reducing power. Based in these two methods, *T. pulegioides* decoction was the most effective extract (EC_{50}=9.5 and 30.7 µg/mL, respectively), opposing to that of *T. caespititius* origin (EC_{50}=13.8 and 39.3 µg/mL, respectively). In addition, *T. herba-barona, T. pseudolanuginosus* and *T. caespititius* aqueous extracts evaluated by β-carotene bleaching assays, presented clearly different results, because β-carotene was inhibited only by *T. pseudolanuginosus* and *T. caespititius* (EC_{50}=2.4 and 6.1 µg/mL, respectively), while *T. herba-barona* was ineffective.

Concerning the eight *Salvia* decoctions targeted in this study, these also possessed high antioxidant activity, as estimated by distinct methods. Based in the DPPH$^{\bullet}$ assay, *S. africana, S. mexicana, S. officinalis 'Icterina'* and *S. elegans* showed promising results (EC_{50}= 6.6-10.7 µg/mL), while *S. africana*, *S. elegans* and *S. mexicana* (EC_{50}= 21-34 µg/mL) were more promising with respect to the ability to reduce Fe^{3+}.

Among the three plants *S. officinalis, S. elegans,* and *S. greggii*, decoctions of *S. elegans* had the highest antioxidant potential for $NO^{\bullet}$ and $O_2^{\bullet-}$ tests, while *S. officinalis* was the most potent in xanthine oxidase and showed slightly better results in ORAC assay. In respect to lipid peroxidation events, *S. apiana* decoction presented superior antioxidant capacity than that the other sage species (EC_{50}= 2.8 and 41 µg/mL in TBARS inhibition and β-carotene bleaching assays, respectively).

Third conclusion: Among *T. herba-barona, T. pseudolanuginosus,* and *T. caespititius* decoctions, that of *T. caespititius* revealed the highest anti-inflammatory potential, showing high ability to scavenge $NO^{\bullet}$ and to inhibit the activity of LOX (EC_{50}= 230 and 591 µg/mL, respectively).

In addition, as evidenced in LPS-activated RAW 264.7 macrophages, *Salvia* decoctions were able to decrease the production of $NO^{\bullet}$ in the order *S. africana* and *S. apiana* (EC_{50}= 48 and 50 µg/mL, respectively) > *S. officinalis 'Icterina'* > *S. mexicana* > *S. farinacea* var. *victoria blue*.

Fourth conclusion: All the *Thymus* and *Salvia* decoctions also exhibited a relevant antibacterial effect against a panel of five bacteria, with promising results observed towards Gram-positive bacteria, especially *S. aureus* (MIC and MBC values between 0.6 and 6 mg/mL, respectively). Concerning Gram-negative bacteria, decoctions of *S. apiana*, *S. officinalis 'Icterina', S. africana, T. zygis* and *T. herba-barona* presented inhibitory and lethal activities against *S. typhimurium,* with the lowest MIC and MBC values (3 to 5 mg/mL). *T. herba-barona, T. pseudolanuginosus* and *T. caespititius* inhibited the growth of Gram-negative bacteria *E. coli* and *P. aeruginosa* (MIC= 5 to 7 mg/mL), while *S. apiana*, *S. officinalis 'Icterina', S. officinalis, S. elegans* and *S. mexicana* presented inhibitory and lethal capacity towards these strains (both MIC and MBC values of 2.8, 7.5, 8.0, 8.5 and 9.5 respectively).

Fifth conclusion: *S. apiana, S. farinacea* var. *victoria blue, S. africana, S. officinalis 'Icterina'* and *S. mexicana* decoctions exhibited cytotoxicity activity towards tumoral cell lines, particularly hepatocellular HepG2, cervical HeLa and breast MCF-7 carcinoma cells. The anti-proliferative activity of these extracts were tumor-selective, as evidenced by comparatively low GI_{50} values in the cancer cell lines (41-89 µg/mL) to those of non-tumor liver cells (297 - 362 µg/mL). Overall, these sage extracts have less cytotoxic effect to non-small lung cancer NCI-H460 cell line.

Sixth conclusion: Decoctions of *S. elegans*, *S. officinalis* and *S. greggii* presented were active against key digestive enzymes of relevance diabetes and obesity. *S. elegans* displayed the best ability to counteract α-glucosidase activity, followed by *S. officinalis*

and *S. greggii*. Possibly, this activity might be correlated to its richness in caffeic acid and its derivatives.

Seventh conclusion: Aqueous extracts of *T. zygis, T. pulegioides* and *T. fragrantissimus* have important nutritional value, being particularly distinguished for their high K^+/Na^+ ratio, richness in polyunsaturated fatty acids and low omega-6/omega-3 fatty acids ratio, which are valuable features of a healthy diet.

Global conclusion:

Salvia and *thymus* decoctions are good sources of phenolic compounds, mostly phenolic acids and flavonoids. The results highlighted the biological activities of the plant extracts under study, including antioxidant, anti-inflammatory and antibacterial properties. In addition, the cytotoxic effects against tumor cells, the metabolic enzyme activity and the nutritional composition of some sage and/or thymus species emphasizes their potential utility for the development of new agents, with application in pharmaceutical and/or food industries.