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Polyphenol composition and biological activity of *Thymus citriodorus* and *Thymus vulgaris*: Comparison with endemic Iberian *Thymus* species

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ABSTRACT

The polyphenol compositions of *Thymus* × *citriodorus* and *Thymus vulgaris* extracts as obtained by exhaustive hydroethanolic (HE) extraction and aqueous decoction (AD) were compared. In addition, their compositions and bioactivities were compared to those of *Thymus pulegioides* and *Thymus mastichina*, grown under the same edaphoclimatic conditions, and *Thymus carnosus*. Rosmarinic acid was the most abundant polyphenol followed by luteolin-hexuronide, salvianolic acids I and K. Cluster analysis suggests a similarity of the polyphenol composition of *T. citriodorus* and *T. vulgaris*. A significant antioxidant activity was observed and correlated with their polyphenol levels. The same being observed for the higher anti-proliferative activity/cytotoxicity of HE extracts on Caco-2 and HepG2 cells as compared to AD extracts. Significant association between the total phenolic compounds with the anti-proliferative activity, for both cell lines, was observed. These results support the importance of salvianolic acids levels in *Thymus* extracts and their *in vitro* anti-proliferative/cytotoxic activities.

1. Introduction

In recent years, a growing interest in phenolic compounds as natural ingredients for food additives and health promoters has been noted and, as a result, there has been a great deal of interest in further investigation and characterization of various traditionally used plant species. Plants from the *Lamiaceae* family, such as the genus *Thymus* L., have been suggested to contribute to several potential health benefits by their reported anti-inflammatory (Khouya et al., 2015) and anti-proliferative (Martins-Gomes, Souto, Cosme, Nunes, & Silva, 2019; Taghouti et al., 2018) as well as anti-microbial (Leal et al., 2017; Nabavi et al., 2015) and anti-oxidant (Kindl, Blazekovic, Bucar, &

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Vladimir-Knezevic, 2015) potentials, with the latter two being also suitable to increase food shelf life. Concerning *Thymus* plants, several species are widely used as culinary herbs, condiments (e.g. *Thymus vulgaris* L.), herbal medicinal products, herbal teas or syrups (e.g. *Thymus* × *citriodorus* (Pers.) Schreb., *Thymus pulegioides* L., *Thymus mastichina* L.), based on their organoleptic, preservative and medicinal properties (e.g. (O. R. Pereira, Macias, Perez, Marin, & Cardoso, 2013; Taghouti et al., 2018).

Thymus × *citriodorus* (Pers.) Schreb. (*T. citriodorus*) or lemon-scented thyme is considered to be a hybrid between *T. pulegioides* and *T. vulgaris* (Lundgren & Stenhagen, 1982). *T. citriodorus* is native from Southern Europe, cultivated in the Mediterranean area and, due to its lemon flavour, is mainly used for culinary purposes, either as seasoning or as herbal tea (Rita, Pereira, Barros, & Ferreira, 2018). Several bioactivities have been attributed to this species, namely anti-oxidant and cytoprotective effects (Pereira et al., 2013) as well as anti-bacterial and anti-radical scavenging activity (Sacchetti et al., 2005). These bioactivities might be intrinsically correlated with its chemical composition, although data to support this are still scarce. The reported chemical composition of non-volatile extracts of *T. citriodorus* includes rosmarinic acid, as the major compound, cinnamic acid derivatives, such as caffeic acid, and derivatives of luteolin, eriodictyol, quercetin, chrysœriol, and apigenin (Pereira, Peres, Silva, Domingues, & Cardoso, 2013; Rita et al., 2018).

Thymus vulgaris L. (*T. vulgaris*) or common thyme is the most often used and studied species within the *Thymus* genus. *T. vulgaris* is an herbaceous, perennial aromatic and medicinal plant, commonly consumed as herbal infusion and as a condiment and spice (flavouring agent) (Pereira et al., 2016). *T. vulgaris* is mostly cultivated for food, pharmaceutical, and cosmetic industries and it is listed in current editions of European Pharmacopoeia, US Pharmacopeia, and in other official papers (Gavarić et al., 2015). Its essential oil is characterized by a high chemical polymorphism, although thymol/carvacrol chemotypes are the most often reported ones (Thompson, Chalchat, Michet, Linhart, & Ehlers, 2003). Apart from essential oils, *T. vulgaris* is a rich source of bioactive compounds such as rosmarinic acid and its derivatives, with rosmarinic acid being reported as the main component (Chizzola, Michitsch, & Franz, 2008; Pereira et al., 2016) along with luteolin, apigenin, caffeic acid and their derivatives, and eriodictyol (Pacifico et al., 2016; Pereira et al., 2016). Several *in vitro* bioactivities of different *T. vulgaris* extracts (prepared with different solvents and methods) have been reported, such as anti-oxidant and DNA-protective activity against oxidation, anti-microbial (Kozics et al., 2013; Martins et al., 2015; Nikolić et al., 2014), anti-inflammatory (Vigo, Cepeda, Gualillo, & Perez-Fernandez, 2004), and anti-tumoral potentials (Pacifico et al., 2016).

Although there is already a basic chemical profile of these species, an in-depth compositional analysis based on an exhaustive extraction (e.g. hydroethanolic exhaustive extraction (Martins-Gomes et al., 2018)) is required. Also, a correlation between chemical composition and bioactivities is needed for further applications as functional foods and/or as sources of bioactive ingredients. The importance of functional foods, nutraceuticals, and other natural health products has been well recognized in connection with health promotion and disease risk reduction (Shahidi, 2009). Functional foods are similar in appearance to conventional foods being consumed as part of the normal diet with demonstrated physiological benefits and can reduce the risk of chronic disease beyond basic nutritional functions, including maintenance of gut health (Food and Agriculture Organization of the United Nations (FAO), authors Report on Functional Foods, Food Quality and Standards Service (AGNS) 2007 [(accessed on 21 March 2020)]). A nutraceutical is "a food or part of a food that provides benefits health in addition to its nutritional content" (Daliu, Santini, & Novellino, 2019). Thus the consumption of these *Thymus* species (*T. vulgaris* and T. *citriodorus*), either fresh or dried, as condiments, herbal teas (widely sold by several companies) or in other preparations, constitute a source of molecules with potential health promoting effects.

Therefore, this study aimed to characterize the chemical composition of two extracts of *T. citriodorus* and of *T. vulgaris*, one obtained by aqueous decoction and the other one by exhaustive hydroethanolic extraction. Furthermore, the present study aimed to deepen the scientific knowledge regarding phenolic profile, antioxidant activity by using three antioxidant methods, and *in vitro* anti-proliferative activity using Caco-2 (human colon adenocarcinoma cell line) and HepG2 (human hepatocellular carcinoma cell line) cells. These cell lines were chosen because this model mimics (as an *in vitro* approach) the effect of plant components' interaction with intestinal tract tissues during absorption and with hepatic tissues, as a result of the first-pass effect of absorbed components.

2. Materials and methods

2.1. Standards and reagents

Commercial standards of salvianolic acid A, apigenin, rosmarinic acid, catechin, luteolin, and ursolic acid were purchased from Sigma-Aldrich/Merck (Algés, Portugal). Caffeic acid and eriodyctiol-(7)-*O*-hexoside were obtained from Extrasynthese® (Genay, France). Oleanolic acid was obtained from Santa Cruz Biotechnology Inc (Frilabo, Porto, Portugal). Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, penicillin, streptomycin, versene, L-glutamine, Trypsin-EDTA, and foetal bovine serum (FBS) were obtained from Gibco (Alfagene, Lisboa, Portugal). Alamar Blue® was obtained from Invitrogen, Life-Technologies (Alfagene, Lisboa, Portugal). Methanol, ethanol, formic acid, and acetic acid were HPLC or MS grade according to the analysis and were purchased from Sigma-Aldrich/Merck (Algés, Portugal). Sodium nitrite, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), sodium nitroprusside, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, potassium persulfate, Folin-Ciocalteu's reagent, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, aluminum chloride (III), sodium molybdate, trichloroacetic acid (TCA), thiobarbituric acid (TBA), 2-deoxy-D-ribose, and hydrogen peroxide (30% solution) were obtained from Sigma-Aldrich/Merck (Algés, Portugal). Other salts and reagents not mentioned were obtained from Sigma-Aldrich/Merck (Algés, Portugal).

2.2. Plant material

T. citriodorus and *T. vulgaris* aerial parts (upper part of stems and their leaves) were kindly supplied by ERVITAL® (Plantas Aromáticas e Medicinais, Lda; Mezio, Viseu, Portugal at 40°58′47.4″N 7°53′43.3″W). Plants were harvested in the production fields of ERVITAL, from the plants used by ERVITAL for the production of herbal infusions and condiments for human consumption. Small portions from fresh plants (50 to 75 plants), grown under organic farming practice, were harvested in October 2014 (post-blooming, end fructification stage). A sample of each plant material was used for authentication by the Botanical Garden office at the University of Trás-os-Montes and Alto Douro (UTAD, Vila Real, Portugal), and a voucher specimen was deposited (HVR22054 to *Thymus vulgaris* L. and HVR21489 to *Thymus* × *citriodorus* L.). Immediately after the harvest, plants were rinsed with distilled water, weighted, and frozen (− 20 °C). After lyophilization (Dura Dry TM μP freeze-drier; − 45 °C and 250 mTorr), the samples were properly stored until further extraction and analysis.

2.3. Preparation of extracts

The lyophilized aerial parts of *T. citriodorus* and *T. vulgaris* were ground to a very fine powder (using a coffee mill) and extracted according to two extraction methods: aqueous decoction (AD), aiming to

mimic human consumption as herbal tea, infusion or condiment, and exhaustive hydroethanolic (HE) extraction, a method optimized to obtain all the extractable compounds within the plant material, as detailed in Martins-Gomes et al. (2018). In both extraction methods, 0.5 g of plants material (lyophilized and ground) were used. AD was performed by adding distilled water (150 mL) to the plant material; the mixture was heated up and boiled, under agitation, for 20 min. After cooling, the volume was reduced to one-third by concentration in a rotary evaporator (35 °C), and the extracts were then lyophilized, weighed to calculate the yields and properly stored until further analysis. HE exhaustive extraction was performed as a three-step sequential extraction. To the plant material, 50 mL of an 80% ethanol solution (v/v, in water) were added. The mixture was agitated for 1 h (orbital shaker, 150 rpm, room temperature) and then centrifuged (7000 rpm, 4 °C; for 5 min (Sigma Centrifuges 3–30 K, St. Louis, MO, USA)). The supernatant was filtered and collected, followed by the addition of another 50 mL of 80% ethanol to the pellet. The extraction was repeated three times, and all supernatants were combined. In both extraction methods, the extracts were filtered twice (first through Whatman n° 4 filter and then through 1.2 μm fiberglass filter (VWR International Ltd., Alfragide, Portugal)) and then concentrated in a rotary evaporator (35 °C), also aiming to remove the ethanol used in HE extraction. Extracts were then lyophilized, weighed to calculate the yields and properly stored until further analysis.

2.4. Total phenolic compound content

The Folin-Ciocalteau method was used to assess the total content of phenolic compounds (TPC). To *T. citriodorus* and *T. vulgaris* extracts (1 mL; 0.1 mg/mL) were added 0.5 mL of Folin-Ciocalteau reagent, 1 mL of sodium carbonate (Na_2CO_3; 7.5%, w/v), and the volume was adjusted to 10 mL with distilled water. The mixture was incubated for 1 h at room temperature, and the absorbance was read at 725 nm using a spectrophotometer (PerkinElmer, Lambda 25 UV/VIS Spectrometer) (Ferreira, Silva, & Nunes, 2018; Machado, Felizardo, Fernandes-Silva, Nunes, & Barros, 2013). Caffeic acid was used as standard, and TPC was expressed as caffeic acid equivalents (mg CA eq/g lyophilized plant or mg CA eq/g extract).

2.5. Total flavonoid content

Total flavonoid content (TFC) was determined as described by Jia, Tang, and Wu (1999), using the aluminum chloride ($AlCl_3$) colorimetric method. To *T. citriodorus* and *T. vulgaris* extracts solution (1 mL; 0.5 mg/mL), 150 μL of an aqueous sodium nitrite solution ($NaNO_2$; 5%, w/v) was added. After a short incubation (5 min, room temperature), 150 μL of an $AlCl_3$ solution (10%, w/v) was added. After 6 min incubation, 1 mL of a sodium hydroxide solution (NaOH; 1 M) was added, and the absorbance was read at 510 nm. Catechin was used as standard, and TFC was expressed as mg catechin equivalents (mg C eq/g lyophilized plant or mg C eq/g extract).

2.6. Total ortho-diphenol content

Ortho-diphenol (ODP) content was determined as described by Machado et al. (2013), using the sodium molybdate colorimetric method. To *T. citriodorus* and *T. vulgaris* extracts (4 mL; 0.1 mg/mL), 1 mL of sodium molybdate solution (Na_2MoO_4; 5%, w/v) was added. The mixture was incubated (15 min, room temperature), and the absorbance was measured at 370 nm. Caffeic acid was used as standard, and ODP content was expressed as mg caffeic acid equivalents (mg CA eq/g lyophilized plant or mg CA eq/g of extract).

2.7. In vitro antioxidant activity assessment

2.7.1. ABTS radical cation ($ABTS^{+\bullet}$) scavenging assay

$ABTS^{+\bullet}$ scavenging assay was performed as described by Machado et al. (2013). To produce $ABTS^{+\bullet}$, equal volumes of ABTS solution (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; 7 mM in water) and potassium persulfate ($K_2S_2O_8$; 2.45 mM in water) were mixed and allowed to react for 15–16 h (in the dark, room temperature). The mixture was diluted using acetate buffer (20 mM, pH 4.5) to obtain an absorbance of 0.700 ± 0.02 (at 734 nm). To assess the scavenging activity of *T. citriodorus* and *T. vulgaris* extracts, $ABTS^{\bullet+}$ solution (2 mL) was added to extracts (200 μL; 0.1 mg/mL), followed by 15 min of incubation, after which the absorbance was read at 734 nm. Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a standard antioxidant. The scavenging potential was expressed as Trolox equivalents (mmol Trolox/g lyophilized plant or mmol Trolox/g extract).

2.7.2. Hydroxyl radicals scavenging assay

Hydroxyl radical ($^{\bullet}OH$) scavenging activity was performed as described by Taghouti et al. (2018). Equal volumes (100 μL) of deoxyribose (20 mM), iron (II) chloride ($FeCl_2$; 1 mM), ascorbic acid (1 mM), and hydrogen peroxide (H_2O_2; 10 mM) were added to 0.5 mL of *T. citriodorus* and *T. vulgaris* extract solutions (0.1 mg/mL), followed by the addition of 400 μL of phosphate buffer solution (20 mM; pH 7.4). A second set of samples was prepared following the protocol described above, but with the addition of EDTA (100 μL; 1 mM). After 1 h of incubation at 37 °C, 1.5 mL of TBA (5%, in 10% TCA) were added, followed by 15 min of incubation at 100 °C. Absorbance was read at 532 nm. A blank (same mixture as described above with, however, distilled water (0.5 mL) replacing the extract solutions) was used as control. The $^{\bullet}OH$ scavenging activity was expressed as percentage inhibition using equation 1 (Eq. 1):

$$Inhibition\ (\%) = \frac{\text{Blank(abs)} - \text{Sample(abs)}}{\text{Blank(abs)}} \times 100 \quad \text{(Eq. 1)}$$

2.7.3. Nitric oxide radical scavenging assay

Nitric oxide radical ($NO^{\bullet}$) scavenging activity was performed as described by Sreejayan and Rao (1997). $NO^{\bullet}$ was produced from a sodium nitroprusside solution (5 mM; in phosphate buffer (0.1 M H_3PO_4; pH 7.4)) that was oxygenated by purging with air for 15 min. To *T. citriodorus* and *T. vulgaris* extracts (0.5 mL; 1 mg/mL), 4.5 mL of sodium nitroprusside solution was added, and the mixture was incubated for 2 h at 35 °C. $NO^{\bullet}$ was then quantified by using the Griess colorimetric assay. To 1 mL of the previous mixture (sample + sodium nitroprusside solution) 1 mL of Griess reagent (equal volumes of 1% sulfanilamide (in 5% H_3PO_4) and 0.1% *N*-alpha-naphthyl-ethylenediamine (in water)) was added; after 3 min of incubation, absorbance was measured at 546 nm. Sodium nitrite was used as the positive control, and $NO^{\bullet}$ scavenging was calculated according to Eq. 1. The scavenging activity was expressed as inhibition percentage. A blank (same mixture as described above with, however, 0.5 mL of distilled water replacing the extract) was used as control.

2.8. Profiling and quantification of individual phenolic compounds by HPLC-DAD and LC-ESI-MS^n

RP-HPLC-DAD and RP-HPLC-ESI-MS^n analyses were carried out as previously described by Ferreira, Silva, Silva, and Nunes (2020) and Taghouti et al. (2018), respectively. Briefly, an Ultimate 3000 HPLC (Dionex, USA) equipped with an Ultimate 3000 pump, a WPS-3000 TSL Analyt auto-sampler, and an Ultimate 3000 column compartment coupled to a PDA-100 photodiode array detector were used for profiling and quantification. Chromatographic separation was performed using a C18 column (ACE 5 C18; 250 mm × 4.6 mm; particle size 5 μm).

Chromeleon software (Version 7.1; Dionex, USA) was used for data acquisition, peak integration, and analysis. LC-ESI-MS^n analysis was carried out using a Thermo Scientific system consisting of a Finnigan Surveyor Plus auto-sampler, photodiode array detector and pump, and an LXQ Linear ion trap detector was used for LC-MS^n analysis. Chromatographic separation was performed with a Luna C18 (2) column (250 mm × 4.6 mm, 5 μm; Phenomenex (Aschaffenburg, Germany)), with temperature kept at 40 °C. Electrospray ionization (ESI) was performed in the negative mode (capillary temperature: 350 °C; capillary voltage: −5 kV; spray voltage: −4 kV). The RP-HPLC-DAD and RP-HPLC-ESI-MS^n program conditions, flow rate, eluents, injection volume and detection parameters were used exactly as described by Taghouti et al. (2018).

Individual phenolic compounds were identified based on UV–VIS spectra, retention time, and mass spectra compared to commercial standards and/or literature data. Calibration curves of available commercial standards were prepared for the quantification of individual phenolic compounds (Taghouti et al., 2018), or using the aglycones or standard compounds with structural similarity when commercial standards were not available. Apigenin-(6,8)-*C*-diglucoside and apigenin-(?)-*O*-hexuronide were quantified as apigenin; Eriodyctiol-(?)-*O*-hexoside was quantified as eriodyctiol-(7)-*O*-hexoside; luteolin-(?)-*O*-hexoside and luteolin-(?)-*O*-hexorunide were quantified as luteolin; salvianolic acid A isomer was quantified as salvianolic acid A; salvianolic acids K and I were quantified as rosmarinic acid.

2.9. Determination of oleanolic acid and ursolic acid in hydroethanolic extracts

The identification and quantification of ursolic (UA) and oleanolic acids (OA) were performed in the HE extracts by RP-HPLC according to the method described previously by Martins-Gomes et al. (2018). Briefly, chromatographic separation was performed using a C18 column (ACE 5 C18; 250 mm × 4.6 mm; particle size 5 μm) using sodium phosphate buffer (30 mM, pH = 3) as solvent A and methanol as solvent B. The volume injected was 50 μL, and the column temperature was kept at 40 °C during the run. Detection was performed at 210 nm. OA and UA identification was performed by comparison with the UV–vis spectrum and retention time of OA and UA commercial standards, and quantification was performed by external calibration.

2.10. In vitro cell viability assay

Two human cell lines were used to evaluate the anti-proliferative/cytotoxic activity of *T. citriodorus* and *T. vulgaris* extracts: Caco-2 (human colon adenocarcinoma cell line; CLS - Cell Lines Service, Eppelheim, Germany) and HepG2 (human hepatocellular carcinoma cell line; ATCC® Number: HB-8065TM, a gift from Prof. C. Palmeira CNC-UC, Portugal). Both cell lines were cultured in DMEM supplemented with 1 mM L-glutamine, 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were maintained at 37 °C in 5% CO_2/95% air with controlled humidity and handled as described by Severino et al. (2014). Stock solutions (10 mg/mL) of *T. citriodorus* and *T. vulgaris* extracts were prepared in PBS for AD extracts and in PBS:DMSO (90:10) for HE extracts. The final concentration of DMSO in test solutions did not exceed 1%.

Anti-proliferative/cytotoxic effects of extracts were evaluated using the Alamar Blue® assay (Andreani, Kiill, de Souza, Fangueiro, Fernandes, Doktorovova, et al., 2014). Briefly, cells suspended in culture media were seeded in 96-well plates (at 5×10^4 cells/mL; 100 μL/well) and were allowed to adhere and stabilize for 48 h. After this period, culture media was replaced by test solutions (100 μL/well), prepared by diluting the respective stock solutions in FBS-free culture medium (test solutions range 50–500 μg/mL). After exposure for 24 h or 48 h, test solutions were removed and replaced by 100 μL/well of Alamar Blue solution (10% (v/v), in FBS-free culture medium). Absorbance was read after 5 h of incubation at 570 nm (reduced form; resorufin) and 620 nm (oxidized form; resazurin), using a Multiskan EX microplate reader (MTX Labsystems; Bradenton, Florida, USA). A control (non-exposed cells) was performed in each assay. Results are expressed as cell viability (% of control), calculated as described by Andreani et al. (2014).

2.11. Statistical analysis

For each extraction method three individual extractions were performed, and the analyses were performed in triplicate for all assays. The IC_{50} values determined for the anti-proliferative activity were calculated as described by Silva et al. (2019). Significant differences between groups were assessed using the non-parametric Kruskal-Wallis method, followed by multiple pairwise comparisons using the Conover-Iman procedure ($\alpha = 0.05$). Correlations were evaluated using Pearson's coefficient (significant if $p < 0.05$) and using the non-parametric Gamma correlation (significant if $p < 0.05$). Graph construction and statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software Inc, California, USA), Microsoft Office Excel Microsoft Corporation, Washington, USA) and Statistica 12.0 (Dell Software, Texas, USA) software.

3. Results and discussion

3.1. Extract yield and chemical composition (Sum Parameters) of extracts

In this study, two extraction methods were selected to obtain *T. citriodorus* and *T. vulgaris* extracts: 1) an exhaustive hydroethanolic (HE) extraction, previously shown to extract 99% of the total extractable compounds in the first three extractions (Martins-Gomes et al., 2018), was chosen as a method to obtain the vast majority of "free" phenolic compounds, allowing to study the full phenolic composition of the selected *Thymus* plants; 2) an aqueous decoction (AD) aiming to mimic the common procedure of beverage preparation for human consumption, as these plants are also used as herbal teas and condiments. For *T. citriodorus*, as expected, the exhaustive HE procedure resulted in higher yields than the one-step AD procedure (~14% vs. ~ 9% (w/w), for HE and AD, respectively, Table 1), but for *T. vulgaris* the yields between the HE and AD extracts were similar, although significantly higher for the AD extraction (Table 1). *T. vulgaris* yields were significantly higher than those obtained for *T. citriodorus*. Compared with yields (same extraction procedures) obtained for other *Thymus* species grown in the same location and harvested in the same year (2014) and time of year (October), i.e. *T. pulegioides* (Taghouti et al., 2018) and *T. mastichina* (Taghouti, Martins-Gomes, Schäfer, Santos, Bunzel, Nunes, et al., 2020) and obtained with the same extraction procedure harvested at the same phenological state (harvested in October), *T. carnosus* (Martins-Gomes et al., 2018), it can be observed (Supplementary material Fig. S.1) that *T. citriodorus* and *T. mastichina* presented lower HE extraction yields than *T. vulgaris*, *T. carnosus*, and *T. pulegioides*; the latter three species presented similar HE extraction yields. *T. vulgaris* presented the highest AD yield (Supplementary material Fig. S.2), and the AD yields followed the order *T. vulgaris* > *T. carnosus* > *T. pulegioides* > *T. citriodorus* ~ *T. mastichina*.

Although *T. vulgaris* showed significantly higher solid yields when compared to *T. citriodorus*, there were no significant differences in the contents of total phenolic compounds when the exhaustive HE extraction was used (Table 1). In fact, when compared to the other *Thymus* species analyzed (Supplementary material Fig. S.3) it can be observed that the TPC levels of *T. citriodorus* and *T. vulgaris* were not significantly different from that observed for *T. mastichina* (Taghouti et al., 2020). However, these three *Thymus* species contained significantly lower levels of TPC when compared to *T. carnosus* (Martins-Gomes et al., 2018) and *T. pulegioides* (Taghouti et al., 2018), with this last species containing the highest levels of TPC (Supplementary Material Fig. S.3). The

Table 1
Extraction yields, chemical composition, and antioxidant activity of *T. citriodorus* (*T. c.*) and *T. vulgaris* (*T. v.*) extracts.

		T. citriodorus		*T. vulgaris*		Extraction method effect		Plant species effect	
		HE	**AD**	**HE**	**AD**	***T. c.***	***T. v.***	**HE**	**AD**
Extraction yield (%, w/w)		14.05 ± 1.47	9.35 ± 1.73	24.34 ± 0.17	25.65 ± 2.14	*	*	*	*
		Chemical composition							
Total phenols	**Ext.**	196.66 ± 2.43	165.14 ± 5.43	103.21 ± 6.07	84.16 ± 6.40	*	*	*	*
(mg caffeic acid equivalent/g)	**D.P.**	27.66 ± 4.57	15.53 ± 4.75	25.12 ± 1.48	21.56 ± 1.64	*	*	n.s.	n.s.
Total flavonoids	**Ext.**	255.93 ± 9.02	282.48 ± 5.57	196.17 ± 8.66	120.33 ± 13.00	*	*	*	*
(mg catechin equivalent/g)	**D.P.**	36.09 ± 5.03	26.51 ± 5.41	47.75 ± 2.11	30.86 ± 3.34	*.	*	n.s.	n.s.
Ortho-diphenols	**Ext.**	176.70 ± 8.65	122.19 ± 36.84	96.16 ± 2.31	62.88 ± 5.77	*	*	*	*
(mg caffeic acid equivalent/g)	**D.P.**	22.97 ± 3.30	16.26 ± 1.92	23.41 ± 0.56	16.13 ± 1.48	*	*	n.s.	n.s.
		Antioxidant activity							
$ABTS^{\bullet+}$	**Ext.**	1.52 ± 0.21	1.21 ± 0.07	0.92 ± 0.03	0.79 ± 0.06	*	*	*	*
(mmol Trolox equivalent)	**D.P.**	0.22 ± 0.05	0.11 ± 0.03	0.22 ± 0.01	0.20 ± 0.01	*	n.s.	n.s.	*
$^{\bullet}OH$ radicals + EDTA									
(% inhibition)			37.97 ± 1.12	–	9.58 ± 1.02	–	–	–	–
$^{\bullet}OH$ radicals –EDTA									
(% inhibition)			30.59 ± 2.08	–	20.71 ± 4.15	–	–	–	–
$NO^{\bullet}$ radicals									
(% inhibition, after 120 min)			41.15 ± 3.64	–	57.61 ± 2.76	–	–	–	–
		Anti-proliferative activity (IC_{50}, µg/mL)							
Caco-2 cells									
	24 h	128.2 ± 5.75	223.70 ± 8.38	> 500	> 500	*	n.s.	*	*
	48 h	114.6 ± 4.38	159.40 ± 16.85	442.45 ± 53.25	376.8 ± 23.20	*	n.s	*	*
HepG2 Cells	**24 h**	> 500	> 500	495.05 ± 11.55	> 500	n.s	n.s.	n.s.	n.s.
	48 h	> 500	> 500	254.25 ± 4.05	> 500	n.s	*	*	n.s.

Results are presented as mean ± standard deviation (n = 3). Abbreviations: AD: aqueous decoction; HE: hydroethanolic extraction; Ext.: extract. D.P.: dry plant. In antioxidant activity, percentage of inhibition obtained for 1 mg/mL of extract. Stat.: non-parametric Kruskal-Wallis method, followed by multiple pairwise comparisons using the Conover-Iman procedure if ($p < 0.05$) were denoted as (*).

(unspecific) sum parameter TPC includes flavonoids, too, which have been measured here as TFC. Levels of TFC obtained by exhaustive HE extraction were not significantly different between *T. citriodorus* and *T. vulgaris* (Table 1). Contrarily to the TPC levels, the TFC levels did not statistically differ among *T. pulegioides*, *T. carnosus,* and *T. vulgaris*. Generally, *T. mastichina* presented the lowest TFC value, followed by *T. citriodorus* (Supplementary Material Fig. S.4). Phenolic compounds containing *ortho*-diphenol structural units were measured as ODP. Different from what was seen for the TFC levels, ODP levels in the different *Thymus* species obtained by exhaustive HE extraction (Supplementary Fig. S.5) followed the same trend as observed for the TPC levels, with *T. pulegioids* presenting the highest ODP levels, whereas the other *Thymus* species did not show significantly different ODP values.

The TPC compositions of the various AD extracts did not follow the same trend as observed for the exhaustive HE extracts, as significantly different TPC extraction yields were analyzed for *T. citriodorus* and *T. vulgaris* (Table 1). However, by using AD extraction 86% of the plant TPC were extracted from *T. vulgaris*, whereas only 56% of the plant TPC were extracted from *T. citriodorus* (Table 1). Different TPC extraction yields using either AD or HE were also observed for the other *Thymus* species (Supplementary Material Fig.s S.3 and S.6.). For *T. carnosus*, in line with that observed for *T. vulgaris*, the AD extraction was also able to extract 85% of the TPC present in the plant, while for *T. mastichina* and *T. pulegioides* the AD extraction was only able to extract 51% and 47%, respectively, of the TPC of the plants. These results show that plant TPC is not directly related with the TPC of the AD, and this may be due to the structural features of the phenolic compounds present in each plant or to different structural and morphological features of each plant and their respective cells. The AD obtained from *T. carnosus* contained the highest level of TPC among all *Thymus* species studied (Supplementary Material Fig. S.6), whereas the *T. mastichina* AD contained the lowest TPC level (Supplementary material Fig. S.6.). The ODP levels of the AD extracts from the different *Thymus* species were similar, with the exception of the AD from *T. mastichina*, which contained a significantly lower ODP level (Supplementary material Fig. S.7). Considering the flavonoids extracted by using the AD extraction, TFC contents from *T. pulegioides* were higher than those from the other *Thymus* species with the exception of *T. vulgaris*, although the TFC level of *T. vulgaris* did not statistically differ from *T. carnosus* and *T. citriodorus* (Supplementary Material Fig. S.8). The lowest TFC content was analyzed in the *T. mastichina* AD extract. This is the first work describing the comparative composition of extracts obtained using the same extraction procedures from different *Thymus* species grown in this specific location in Portugal for the 2014 harvesting year (*T. citriodorus, T. vulgaris, T. mastichina and T. pulegioides*) and additionally *T. carnosus* extracts obtained with the same extraction procedure at the same phenological stage, therefore corresponding to specific edaphoclimatic conditions, and hence further work is need to validate these results for other growing locations and harvest years.

3.2. Phenolic compound profiles in aqueous decoction and hydroethanolic extracts

In order to understand whether the extraction and compositional variations, between *T. vulgaris* and *T. citriodorus* (and the other *Thymus* species), described in section 3.1., are reflected by their phenolic profiles and their individual phenolic compounds, the phenolic composition of *T. citriodorus* and *T. vulgaris* was determined by HPLC-DAD and HPLC-MS^n. The phenolic profiles as well as the individual phenolic compounds and their concentrations in the HE extracts from *T. citriodorus* and *T. vulgaris* are shown in Fig. 1 and in Table 2. Table 2 also shows the phenolic compounds and their concentrations in AD extracts of both *Thymus* species. The relative amount of each phenolic compound present in HE and AD extracts determined by HPLC-DAD is consistent with the results obtained by the colorimetric methods discussed above for the TPC, TFC and ODP (Table 1). For both *Thymus* species and both extraction methods, rosmarinic acid was the most abundant phenolic compound (Table 2). Rosmarinic acid represented 54% and 66% of the total phenolic compounds (sum of individual phenolic compounds) extracted by the HE for *T. citriodorus* and *T vulgaris*, respectively. The amount of rosmarinic acid in both *T. citriodorus* and *T. vulgaris* plants was significantly lower than in *T. pulegioides*

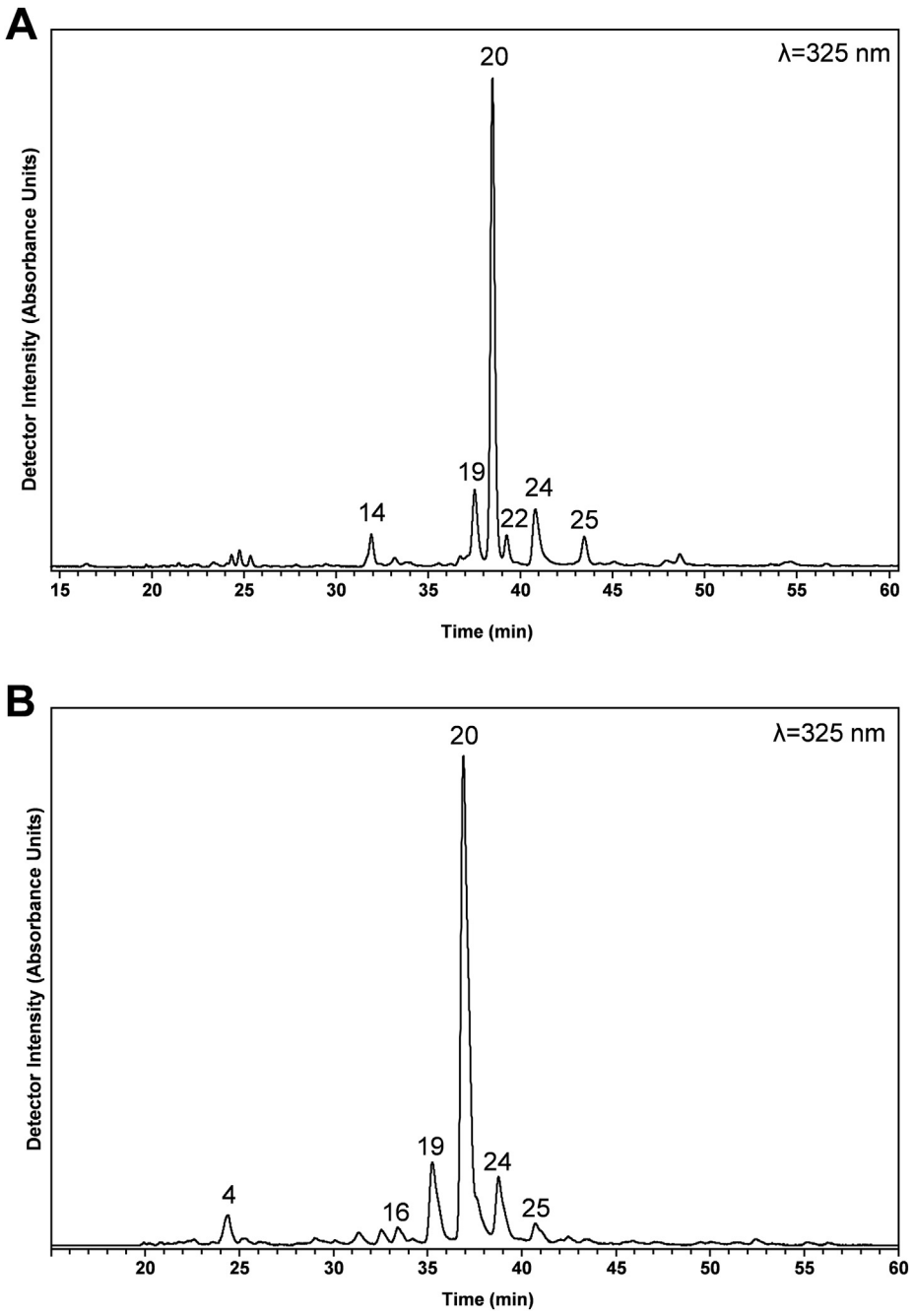

Fig. 1. Phenolic profiles of hydroethanolic (HE) extracts from *T. citriodorus* (A) and *T. vulgaris* (B). For peak identification please refer to Table 2.

plants (Taghouti et al., 2018) but higher than in *T. carnosus* plants (Martins-Gomes et al., 2018) and *T. mastichina* (Taghouti et al., 2020). The share of rosmarinic acid in the total phenolic content (sum of individual phenolics, HE extracts) in *T. vulgaris* (66%) and *T. citriodorus* (54%) is higher than in *T. pulegioides* (35%), *T. mastichina* (34%) and *T. carnosus* (20%). Although other phenolic compounds were quantified in the various extracts, rosmarinic acid was the only phenolic compound present in quantifiable amounts across all five *Thymus* species. Other phenolic compounds that were present in quantifiable amounts in some *Thymus* species extracts were detected by HPLC-MS (Table 2) in the other extracts, too, but the signal-to-noise ratios in the UV-detected chromatograms did not allow for the quantification. Phenolic compounds that were detected in all *Thymus* species but could not be quantified in all species were eriodictyol-hexoside (not quantified in *T. mastichina*), luteolin-hexoside (not quantified in *T. citriodorus*), salvianolic acid I (not quantified in *T. carnosus*) and salvainolic acid K (not quantified in *T. pulegioides*). *T. citriodorus* and T. *vulgaris* did not present quantifiable amounts of eriodictyol-di-hexoside, eriodictoyol-hexorunide and naringenin-hexoside (only quantified in *T. pulegioides*), quercetin-hexoside, salvianolic acid B/E isomer 2 and chrysoeriol-hexuronide (only quantified in *T. mastichina*) and luteolin-pentoside-hexoside and salvianolic acid A (only quantified in *T. carnosus*). On the other hand the salvainolic acid A isomer, previously described in *T. carnosus* and *T. mastichina* by our research group, is also present in quantifiable amount in *T. citriodorus* but not in *T. vulgaris*.

Concerning the relative amount of total salvianolic acids and rosmarinic acid to the total flavonoids present in each *Thymus* plant, *T. carnosus* presented the highest ratio (15.2) followed by *T. vulgaris* (5.6), *T. citriodorus* (3.0), *T. mastichina* (2.6), and *T. pulegioides* (0.51). *T. citriodorus* that is a hybrid between *T. pulegioides* and *T vulgaris* presents a

Table 2
Phytochemical composition of hydroethanolic (HE) and aqueous decoction (AD) extracts of *T. citriodorus* (*T. c.*) and *T. vulgaris* (*T. v.*) as determined by HPLC/DAD-ESI/MS.

	Compound	R.T. (min)	ESI-MS^2	*T. citriodorus*		*T. vulgaris*		E.M.E.		P.S.E.	
				mg/g extract							
				HE	**AD**	**HE**	**AD**	***T. c.***	***T. v.***	**HE**	**AD**
1	Salvianic acid A	18.16 ± 0.10	[197]:179	n.q.	n.d.	n.d.	n.d.				
2	Eriodictyol-di-(?)-*O*-hexoside	21.85 ± 0.07	[611]:449;287	n.q.	n.q.	n.q.	n.q.				
3	Naringenin-di-hexoside	22.12 ± 0.07	[595]:433;271	n.q.	n.q.	n.d.	n.d.				
4	Apigenin-(6,8)-*C*-diglucoside	24.63 ± 0.09	[593]:575;503;473;383;353	4.25 ± 0.65	1.78 ± 0.10	n.q.	n.q.	*			
5	Hydroxyjasmonic acid -hexoside	24.82 ± 0.06	[387]:369;225;207;163	n.q.	n.q.	n.q.	n.q.				
6	Caffeic acid	25.08 ± 0.10	[179]:135	0.70 ± 0.43	2.86 ± 0.12	n.d.	n.d.	*		*	*
7	Rosmarinic acid derivative	25.22 ± 0.11	[377]:359;137;267;197;179	n.q.	n.q.	n.d.	n.d.				
8	Eriodictyol-(?)-*O*-hexoside	25.65 ± 0.04	[449]:287	6.05 ± 1.02	3.06 ± 1.85	2.13 ± 0.65	n.q.	*.	*	*	*
9	Unknown	25.67 ± 0.04	[495]:486;451;375;368	n.q.	n.q.	n.d.	n.d.				
10	Prolithospermic acid	28.33 ± 0.04	[357]:313;269;245;203	n.d.	n.q.	n.d.	n.d.				
11	Quercetin-(?)-*O*-hexoside	29.66 ± 0.11	[463]:301	n.q.	n.d.	n.q.	n.q.				
12	Naringenin-(?)-*O*-hexoside	29.98 ± 0.62	[433]:313;271	n.q.	n.q.	n.q.	n.q.				
13	Eriodictyol-(?)-*O*-hexuronide	31.95 ± 0.22	[463]:287;175	n.q.	n.q.	n.q.	n.q.				
14	Luteolin-(?)-*O*-hexoside	32.11 ± 0.10	[447]:285	n.q.	n.q.	n.q.	n.q.				
15	Quercetin-(?)-*O*-hexuronide	33.29 ± 0.15	[477]:301	n.q.	n.d.	n.d.	n.d.				
16	Luteolin-(?)-*O*-hexoside	34.18 ± 0.18	[447]:285	n.q.	n.d.	0.48 ± 0.03	0.95 ± 0.30	n.s.	*	*	*
17	Salvianolic acid B/E isomer 2	36.49 ± 0.37	[717]:555;519;475;357;295	n.d.	n.d.	n.q.	n.d.				
18	Salvianolic acid A isomer	37.33 ± 0.22	[493]:383;313;295	8.32 ± 0.62	11.91 ± 0.96	n.q.	n.q.	*	n.s.	*	*
19	Luteolin-(?)-*O*-hexuronide	37.73 ± 0.29	[461]:285;175	17.25 ± 3.13	13.22 ± 0.36	17.83 ± 1.99	15.66 ± 1.51	*.	n.s.	n.s.	n.s.
20	Rosmarinic acid	38.65 ± 0.22	[359]:223;179;161	86.23 ± 13.88	51.80 ± 1.86	90.48 ± 7.77	22.93 ± 9.51	*	*	n.s.	n.s.
21	Sagerinic acid	38.50 ± 0.22	[719]:359	n.q.	n.d.	n.q.	n.d.				
22	Chrysoeriol-(?)-*O*-hexoside	39.38 ± 0.16	[461]:299;160	11.96 ± 1.83	7.06 ± 0.65	n.q.	n.q.	*	n.s.	*	*
23	Salvianolic acid K	40.81 ± 0.01	[555]:537;493;359	6.17 ± 2.26	5.21 ± 1.54	6.52 ± 0.80	3.56 ± 0.89	n.s.	n.s.	n.s.	n.s.
24	Salvianolic acid I	41.24 ± 0.40	[537]:493;359	25.87 ± 3.90	n.d.	11.61 ± 0.93	n.d.	*	*	*	n.s.
25	Apigenin-(?)-*O*-hexuronide	43.59 ± 0.37	[445]:269;175	n.q.	n.q.	0.58 ± 0.31	n.q.				
26	Chrysoeriol-(?)-*O*-hexuronide	44.79 ± 0.38	[475]:299	n.q.	n.d.	n.d.	n.d.				
27	Salvianolic acid B/E isomer	47.72 ± 0.27	[717]:555;519;475;357;295	n.q.	n.d.	n.d.	n.d.				
		Total phenolic compounds		166.82 ± 27.73	96.91 ± 0.04	129.64 ± 8.72	50.02 ± 2.17	*	*	*	*
		Total flavonoids		39.51 ± 6.64	25.12 ± 1.45	20.11 ± 0.22	16.61 ± 1.81	*	*	*	*
		Total phenolic acids		127.31 ± 21.09	71.79 ± 1.41	108.62 ± 9.36	32.44 ± 1.27	*	*	n.s.	*

Abbreviations: RT: retention time; ESI-MS^2: Fragment ions obtained after fragmentation of the pseudo-molecular ion $[M]^-$; n.q.: not quantified; n.d.: not detected; E.M.E.: extraction method effect; P.S.E.: plant species effect. Stat.: non-parametric Kruskal-Wallis method, followed by multiple pairwise comparisons using the Conover-Iman procedure if ($p < 0.05$) were denoted as (*). Results are presented as content in mg/ gram as mean ± standard deviation, n = 3.

chemical composition much more comparable to *T. vulgaris* than to *T. pulegioides*, concerning both the profile of phenolic compounds and their amounts. In fact, using both the chemical composition of the five *Thymus* plants obtained by exhaustive HE extraction as chemical descriptors, and cluster analysis as a pattern recognition method, the formation of three distinct clusters can be observed: the cluster on the left comprises the *T. carnosus* sample, the middle cluster is only composed of *T. pulegioides,* and the third cluster at the right contains *T. mastichina*, *T. citriodorus*, and *T. vulgaris* (Fig. 2A and C).

A logarithmic relation between the amount of total phenolic compounds extracted by AD extraction and the amount of total phenolic compounds present in the plant (exhaustive HE extraction) was observed (Fig. 2B). A logarithmic relation was also found for the amount of both rosmarinic acid and salvianolic acid K being either extracted with AD or HE. Therefore, this logarithmic relation between the amounts of total phenolic compounds and selected phenolic compounds extracted with AD or HE confirms the differences in extraction yields as observed for the different *Thymus* species discussed previously. However, the chemical nature of the individual compounds strongly affects the AD extractability, too. For example, salvianolic acid I, although present in *Thymus* plants with the exception of *T. carnosus*, was not extracted in quantifiable amounts by using AD (Table 2). Nevertheless, although there are differences among the AD and HE extract compositions, concerning the phenolic compounds present and their contents for each *Thymus*, cluster analysis of the AD and HE (Supplementary Material Fig. S.9) extracts concerning their phenolic composition show that the AD extracts from each *Thymus* are still more similar to the HE extracts obtained from the same *Thymus,* reflecting the impact of *Thymus* plants composition of the AD extract obtained (Supplementary Material Fig. S.10).

3.3. Oleanolic acid and ursolic acid contents

Oleanolic (OA) and ursolic (UA) acids are two triterpenes that are commonly described as being present in the alcoholic extracts from various *Thymus* species, for example, from *T. serpyllum* (3.7 and 13.9 mg/g dry plant, for OA and UA, respectively (Janicsák, Veres, Zoltán Kakasy, & Máthé, 2006), *T. carnosus* (9.9 and 18.7 mg/g dry plant, for OA and UA respectively (Martins-Gomes et al., 2018), and *T. pulegioides* (0.34 and 0.80 mg/g dry plant, for OA and UA respectively (Taghouti et al., 2018). However, these triterpenes were not detected in the HE extracts from *T. vulgaris* and *T. citriodorus* (Table 2), just as previously observed for *T. mastichina* (Taghouti et al., 2020). Nevertheless, Janicsák et al. (2016) reported that *T. vulgaris* and *T. citriodorus*

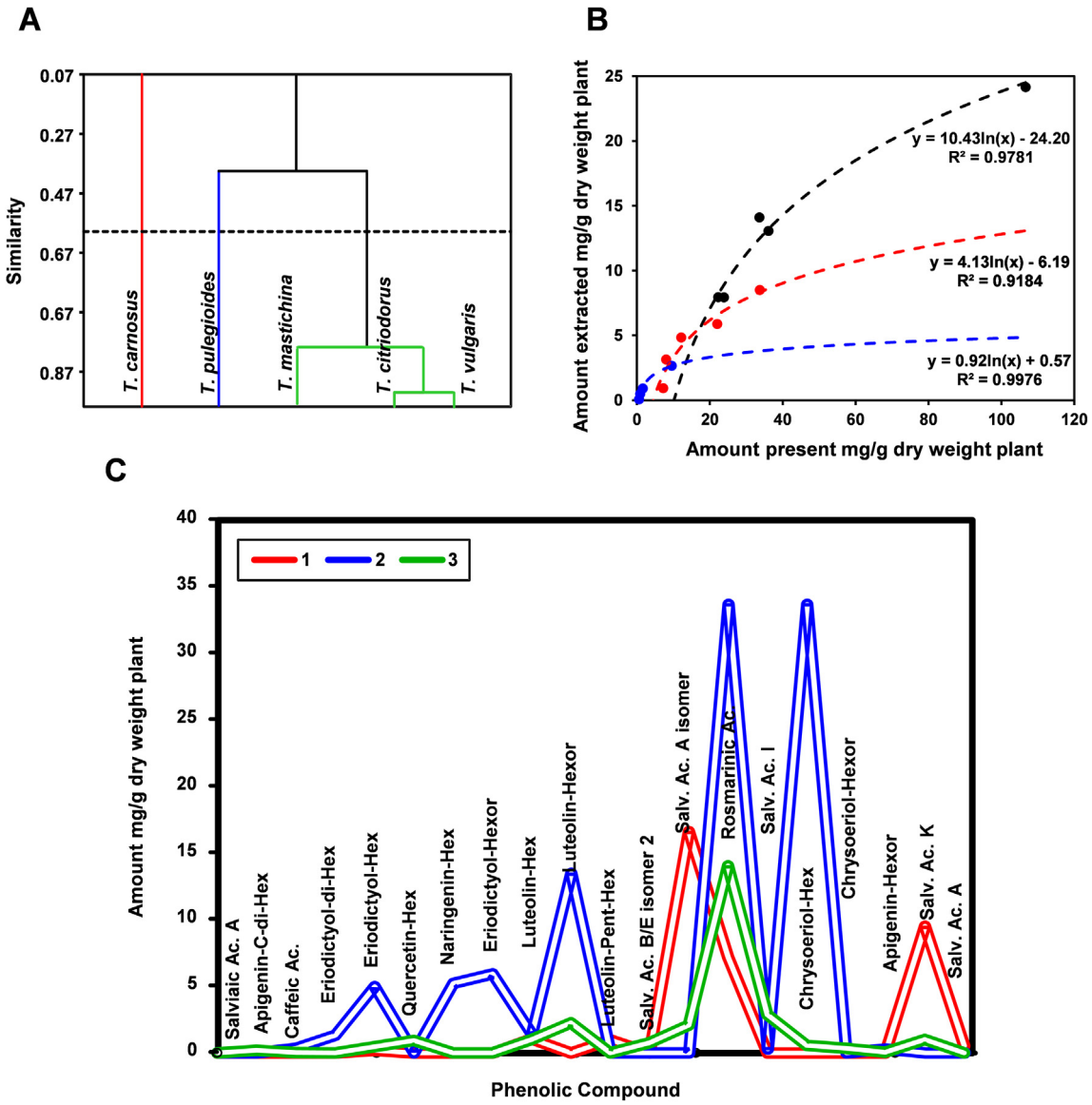

Fig. 2. (A) Dendogram of cluster analysis based on the phenolic composition of hydroethanolic (HE) extracts. (B) Relation between the amount of total phenolic compounds (•), rosmarinic acid (●) and salvianolic acid K (●) extracted by aqueous decoction (AD) (y-axis) and by HE extraction (x-axis) of *Thymus* plants. (C) Average levels of phenolic compounds present in each cluster.

(collected in Hungary; July 1999) extracts, obtained by using Soxhlet extraction with methanol as solvent for 6 h, contained measurable amounts of OA (1.7 and 2.6 mg/g dry weight) and of UA (5.2 and 6.4 mg/g dry weight), respectively to *T. vulgaris* and *T. citriodorus*. These differences may reflect a different phenological state of the plant, the effect of location and climate on chemical composition of plants, and may also result from a different extraction method and solvent.

3.4. *In vitro* antioxidant activity

The extracts of *T. citriodorus* and *T. vulgaris* showed a significant scavenging activity of ABTS radical cations (Table 1). As expected, significantly higher scavenging activity values were obtained for the HE extracts (~1.52 and 0.92 mmol Trolox eq./g extract, respectively; Table 1) as compared to the AD extracts (~1.21 and 0.79 mmol Trolox eq./g extract, respectively, Table 1). The values expressed as Trolox eq./g dry plant (Table 1) were lower than those found by Taghouti et al. (2018)) for HE and AD extracts of *T. pulegioides* (0.15 and 0.34 mmol Trolox eq./g dry plant, respectively), in the same range of those values found for the extracts of *T. carnosus* (0.14 and 0.21 mmol Trolox eq./g dry plant, respectively) (Martins-Gomes et al., 2018) and higher than those observed for *T. mastichina* (0.08 and 0.20 mmol Trolox eq./g dry plant, respectively) (Taghouti et al., 2020). Although a significant correlation between the extracts TPC ($r = 0.827$, $p < 0.00316$), TFC ($r = 0.798$, $p < 0.005663$) and ODP ($p < 0.910$, $p < 0.000257$) and their Trolox equivalent antioxidant capacity (TEAC) was observed, a more detailed analysis of the impact of the individual phenolic compounds present in the extracts on the antioxidant activity showed that rosmarinic acid mostly affects the antioxidant activity presented by the extract ($r = 0.874$, $p < 0.000945$). This is partially explained by the fact that it is the major phenolic compound in most extracts as discussed above. Nevertheless, the contents of other phenolic compounds also showed significant correlations with the observed TEAC values, e.g. eriodictyol-glucoside ($r = 0.759$, $p < 0.0109$), luteolin-hexuronide ($r = 0.734$, $p < 0.0157$), and chrysoeriol-hexoside ($r = 0.771$, $p < 0.00903$). In fact, a significant correlation ($r = 0.878$, $p < 0.000834$) between the plants antioxidant activity with their total phenolic compounds extracted by the two extraction methods, obtained by the sum of the individual phenolic compounds, was observed.

Results from the hydroxyl radical ($^{\bullet}OH$) scavenging assays

demonstrated that AD extracts from *T. citriodorus* inhibit hydroxyl radicals more strongly than AD extracts from *T. vulgaris*. This was found in the absence and in the presence of EDTA in the test system, namely the *T. citriodorus* ˙OH scavenging activity was about 1.5-fold (-EDTA) and 4.2-fold (+EDTA) higher as compared to *T. vulgaris* (Table 1). However, the activity of the *T. citriodorus* extracts (Table 1) was lower than the activity of a methanolic extract of *T. dacicus* (Petrović et al., 2017), which produced 50% of the radical scavenging at 18.85 μg/mL. However, the inhibition percentage of *T. citriodorus* extracts was comparable to AD extracts of *T. carnosus* (Martins-Gomes et al., 2018) and to AD extracts of *T. pulegioides* (Taghouti et al., 2018) and *T. mastichina* (Taghouti et al., 2020).

Concerning the scavenging of NO radical, *T. vulgaris* extracts showed a higher (~1.4-fold) scavenging activity when compared to *T. citriodorus* (41%; Table 1). Similar values of NO radical scavenging activity were observed for AD extracts from *T. carnosus* (40%) (Martins-Gomes et al., 2018) and for AD extracts from *T. pulegioides* (35.76%) (Taghouti et al., 2018) and *T. mastichina* (39%) (Taghouti et al., 2020).

3.5. Anti-Proliferative effect of T. Citriodorus and T. Vulgaris extracts

In order to evaluate the anti-proliferative effect of AD and HE extracts from *T. citriodorus* and *T. vulgaris*, we used the Alamar Blue (AB) reduction assay and two cell lines, HepG2 and Caco-2. Cells were exposed to different concentrations of *T. citriodorus* and *T. vulgaris* extracts (50, 100, 200, and 500 μg/mL) for 24 h or 48 h, and results were compared with those obtained from control cells (non-exposed cells). The effect of both extracts was more pronounced on Caco-2 cells (Fig. 3) than on HepG2 cells (Supplementary Material Fig. S.11). HE extracts presented a higher anti-proliferative activity/cytotoxicity on Caco-2 cells than the AD extracts (Fig. 3), which correlates with their concentration of phenolic compounds (Table 2; TPC HE/AD: 1.7 and 2.6 in *T. citriodorus* and *T. vulgaris*, respectively). On Caco-2 cells, the half-maximal inhibitory concentration (IC_{50}) of HE extracts, at 24 h exposure, was statistically significant lower than the IC_{50} values obtained for the AD extracts of *T. citriodorus* (HE: 128.2 ± 5.75 μg/mL; AD: 223.70 ± 8.38 μg/mL) and *T. vulgaris* (IC_{50} > 500 μg/mL, both extracts). The same trend was observed after 48 h of exposure with the effect of exposure time being evident, as the IC_{50} values were lowered when compared to 24 h of exposure (Table 1). Caco-2 cells were more sensitive to *T. citriodorus* and *T. vulgaris* extracts than HepG2 cells as indicated by statistically significant lower IC_{50} values after 24 h or 48 h of exposure (Table 1) for both extracts. The IC_{50} values obtained for the treatment of Caco-2 cells with HE and AD extracts from *T. vulgaris* were higher than those observed for the treatment with *T. citriodorus* extracts (Table 1) and also higher than those reported for the HE and AD extracts from *T. carnosus* (Martins-Gomes et al., 2019), *T. pulegioides* (Taghouti et al., 2018), and *T. mastichina* (Taghouti et al., 2020). On the other hand, for the same cell line, IC_{50} values obtained for *T. citriodorus* HE extracts (Table 1) were higher than those reported for *T. carnosus* (Martins-Gomes et al., 2019) and *T. mastichina* (Taghouti et al., 2020) HE extracts, and similar to that reported from *T. pulegioides* (Taghouti et al., 2018). Concerning *T. citriodorus* AD extracts, the IC_{50} values obtained on Caco-2 cells (24 h: 224 μg/mL; 48 h: 159 μg/mL) are lower than those obtained for *T. carnosus* AD extracts (24 h: 510 μg/mL; 48 h: 203 μg/mL (Martins-Gomes et al., 2019)), but higher than those obtained for *T. pulegioides* (24 h: 138 μg/mL; 48 h: 82 μg/mL, (Taghouti et al., 2018)) and *T. mastichina* (24 h: 221 μg/mL; 48 h: 96 μg/mL (Taghouti et al., 2020) AD extracts.

Concerning HepG2 cells, at both exposure times, the IC_{50} values obtained for *T. citriodorus* AD and HE extracts were higher than the experimental concentrations tested in this work (> 500 μg/mL; Table 1), denoting very low cytotoxicity for this cell line. These values are higher than that observed for the HE extracts from *T. vulgaris* (24 h: 495 μg/mL; 48 h: 254 μg/mL (Table 1).The IC_{50} values of *T. vulgaris* AD extracts on HepG2 cells, after 48 h exposure (> 500 μg/mL), higher than those obtained for *T. carnosus* (478 μg/mL (Martins-Gomes et al., 2019) and *T. mastichina* (285 μg/mL (Taghouti et al., 2020), indicate

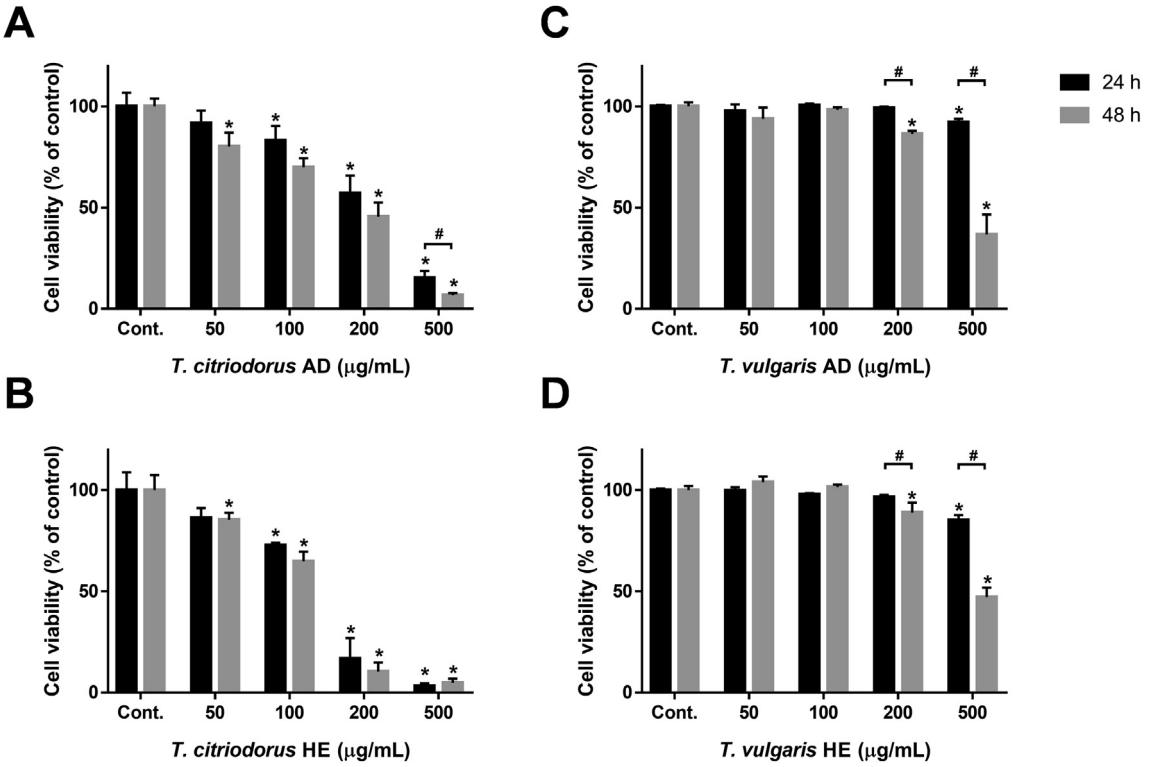

Fig. 3. Effects of aqueous decoction (AD) and hydroethanolic extracts (HE) of *T. citriodorus* (**A**: AD; **B**: HE) and *T. vulgaris* (**C**: AD; **D**: HE) extracts in Caco-2 cells after 24 h and 48 h of exposure. Results are expressed as (mean ± SD, n = 4). Statistical significant differences ($p < 0.05$) between control and sample concentrations at respective incubation time are denoted by *, and between concentration periods at same concentration are denoted by #.

Table 3
Association between the phenolic compounds present in aqueous decoction and hydroethanolic extracts from the five *Thymus* species and the IC_{50} values observed for Caco-2 and HepG2 cell lines exposed for 24 and 48 h to the extracts, using the Gamma non-parametric correlation .

	Caco-2		HepG2	
	24 h	48 h	24 h	48 h
Total phenolic compounds[a]	−0.511	−0.467	−0.543	
Total salvianolic and rosmarinic acids	−0.600	−0.467	−0.771	−0.571
Quercetin-(?)-*O*-hexoside		−0.647	−0.538	−0.647
Luteolin-(?)-*O*-pentoside-hexoside				−0.529
Salvianolic acid B/E isomer 2		−0.647	−0.538	−0.647
Salvianolic acid A isomer				−0.538
Salvianolic acid I			−0.478	
Chrysoeriol-(?)-*O*-hexuronide		−0.647	−0.538	−0.647
Oleanolic acid	−0.647	−0.647	−0.692	−0.647
Ursolic acid	−0.647	−0.647	−0.692	−0.647

[a] sum of individual phenolic compounds determined by HPLC.

low cytotoxicity on this cell line.

In order to explore the possible associations between the chemical composition of the extracts of the five *Thymus* species obtained by the two extraction methods (HE and AD) and the observed ant-proliferative/cytotoxic activity of these extracts against Caco-2 and HepG2 cell lines for the two exposure times, evaluated by their IC_{50} values, a non-parametric gamma correlation was performed. As can be observed in Table 3, there is a significant association between the total phenolic compounds (obtained by summing up the individual phenolic compounds) present in the extracts and the IC_{50} values of the extracts studied for both cell lines, although, in case of the HepG2 cell line, this is only true for the IC_{50} values determined after 24 h. On the other hand, the Total Salvianolic Acids content of the extracts also showed a significant association to the observed IC_{50} values for both cell lines and exposure times. Oleanolic and ursolic acids also showed a significant association between their relative content and the observed IC_{50} values for both cell lines and exposure times. Among the individual phenolic compounds in the extracts, quercetin-hexoside, salvianolic acid B/E isomer 2, and chrysoeriol-hexuronide show a significant association to the IC_{50} values obtained for the Caco-2 cell line after 48 h exposure (but not for 24 h of exposure) and for the HepG2 cell line for both times of exposure. These results support the importance of the salvianolic acid composition of the *Thymus* extracts obtained for the different *Thymus* species and their anti-proliferative/cytotoxic activity, although the flavonoids present can also have a significant influence on the anti-proliferative/cytotoxicity of the *Thymus* extracts.

Wang, Wu, Tao, Liu, and El-Nezami (2011) observed that salvianolic acid B induced a time- and dose-dependent reduction on HepG2 cell proliferation. Salvianolic acid B, at the highest tested concentration (250 μM), induced a reduction on cell proliferation of 75% and 80%, after 24 and 48 h of exposure, respectively (Wang et al., 2011). In another study, salvianolic acid B inhibited the growth of several head and neck squamous carcinoma cell lines (JHU-022 and JHU-013 cells), with IC_{50} values of 18 μM and 50 μM, respectively (Hao, Xie, Korotcov, Zhou, Pang, Shan, et al., 2009). Also, salvianolic acid B at a concentration of 125 μM reduced the cell viability of different human cancer cell lines, namely, liver cell lines, Huh-7 and SK-HEP-1, to 45% and to 25% of the control (Hao et al., 2009). Salvianolic acids A and B have been reported as good candidates against several types of cancer as these molecules target several cell mechanisms involved in apoptosis, cell cycle regulation, and inflammation (Hao et al., 2009; Qin, Rasul, Sarfraz, Sarfraz, Hussain, Anwar, et al., 2019). This is because salvianolic acids act through mechanisms that modulate various signaling pathways (e.g., mitogen activated protein kinase (MAPK), phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/PKB/Akt), nuclear factor kappa B (NF-κB), mammalian target of rapamycin (mTOR) pathways), which are often deregulated in cancer cells and are usually associated with drug resistance (Qin et al., 2019). Nevertheless, the nature of salvianolic acid derivatives present in the extracts can also influence the anti-proliferative activity, as shown here by the significant associations of some individual salvanolic acids and the anti-proliferative/cytotoxic activity of the extracts obtained from the different *Thymus* species (Table 3).

4. Conclusions

This is the first work describing the detailed phenolic composition of exhaustive hydroethanolic (HE) and aqueous decoction (AD) extracts of *T. vulgaris* and *T. citriodorus*, grown under the same conditions and collected at same phenological stage. The phenolic composition of each extract was correlated with its observed *in vitro* bioactivities. Among other common phenolic compounds normally present in *Thymus* species, salvianolic acids K, I and A isomer were identified. Extracts of *T. vulgaris* and *T. citriodorus* showed a significant scavenging activity of $ABTS^{\bullet+}$, $^{\bullet}OH$, and $NO^{\bullet}$. The HE extract from *T. citriodorus* showed the best anti-proliferative activity against Caco-2 cells while *T. vulgaris* extracts showed low and very-low cytotoxicity against Caco-2 and HepG2 cells, respectively. *T. vulgaris* and *T. citriodorus* polyphenol composition and bioactivities were compared to those of *T. pulegioides*, and *T. mastichina*, grown under the same edaphoclimatic conditions. Cluster analysis supports that *T. citriodorus* polyphenol composition is more similar to *T. vulgaris* than to *T. pulegioides*. A significant association between the total phenolic compounds, obtained by summing up the individual phenolic compounds, and the individual compounds present in the extracts, namely salvianolic acid B/E isomer 2, A isomer and I as well as total salvianolic acids and rosmarinic acid, with the anti-proliferative/cytotoxicity of both cell lines was observed when considered the results obtained from the five *Thymus* species. These results support the importance of the levels of salvianolic acids in *Thymus* extracts and their anti-proliferative/cytotoxic activity.

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CRediT authorship contribution statement

Meriem Taghouti: Investigation, Data curation, Formal analysis, Writing - original draft. **Carlos Martins-Gomes:** Investigation, Writing - original draft, Data curation, Formal analysis, Writing - review & editing. **Luís M. Félix:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Judith Schäfer:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Joao A. Santos:** Supervision, Writing - review & editing. **Mirko Bunzel:** Methodology, Resources, Investigation, Writing - review & editing. **Fernando M. Nunes:** Conceptualization, Project administration, Methodology, Resources, Supervision, Formal analysis, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Amélia M Silva:** Conceptualization, Project administration, Methodology, Resources, Supervision, Formal analysis, Data curation, Visualization, Writing -

original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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