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Orange thyme: Phytochemical profiling, *in vitro* bioactivities of extracts and potential health benefits

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A R T I C L E I N F O

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Chemical compounds:
Apigenin (PubChem CID: 5280443)
Eriodictyol (Pubchem CID 440735)
Luteolin (PubChem CID: 5280445)
Rosmarinic acid (PubChem CID: 5281792)
Salvianolic acid I (Pubchem CID 10459878)
Salvianolic acid K (Pubchem CID 10482829)

A B S T R A C T

Orange thyme (*Thymus fragrantissimus*) is becoming widely used in food as a condiment and herbal tea, nevertheless its chemical composition and potential bioactivities are largely unknown. Thus the objective of this work is to obtain a detailed phytochemical profile of *T. fragrantissimus* by exhaustive ethanolic extraction and by aqueous decoction mimicking its consumption. Extracts showed high content in rosmarinic acid, luteolin-*O*-hexuronide and eriodictyol-*O*-hexuronide; these were the main phenolic compounds present in orange thyme accounting for 85% of the total phenolic compounds. Orange thyme extracts presented high scavenging activity against nitric oxide and superoxide radicals. Both extracts presented significant inhibitory effect of tyrosinase activity and moderate anti-acetylcholinesterase activity. Both extracts showed a good *in vitro* anti-inflammatory activity and a weak anti-proliferative/cytotoxic activity against Caco-2 and HepG2 cell lines supporting its safe use. Orange thyme is a very good source of bioactive compounds with potential use in different food and nutraceutical industries.

Introduction

Plants and their constituents have been used as sources of therapeutic agents and as food ingredients since ancient times (Raskin et al., 2002). Nowadays, there is an emerging scientific and commercial interest in medicinal and aromatic plants (MAPs) due to their economic potential and proved biological activities, which are mainly correlated to their secondary metabolites, e.g. terpenoids, alkaloids and phenolics (Leal et al., 2017). However, despite the promising potential of many herbal species, the majority of them have not been comprehensively studied.

Among MAPs, the genus *Thymus* L. (belonging to the Lamiaceae family) originated in the Mediterranean area is currently widespread around the world, comprising about 350 species (Ghasemi Pirbalouti, Emami Bistghani, & Malekpoor, 2015; Silva et al., 2020). *Thymus vulgaris* L. (common thyme) represents one of the most cultivated

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herbaceous and perennial herb of *Thymus* genus and is widely used in food and aroma industry due to its chemical composition and related bioactivities, which have been widely described (e.g. (Martins-Gomes et al., 2018; Martins et al., 2015; Pereira et al., 2016; Taghouti et al., 2018). With the growing search for new flavors and aromas, other *Thymus* species are being introduced in food industry and in human diet, as is the case of orange thyme (also known as orange-scented thyme, *Thymus vulgaris* '*Fragrantissimus*', *Thymus fragrantissimus*, *Thymus* '*Fragrantissimus*', *Thymus vulgaris* var. *fragrantissimus*) due to its highly aromatic orange-scented leaves that enriches culinary recipes. This variety, although not yet botanically recognized as a subspecies, is presumed to be a hybrid of *Thymus vulgaris*. However, to the best of our knowledge, there is few data reporting its chemical composition, bioactivities and toxicity, knowledge that is necessary to ensure safe use in culinary. The antioxidant properties (assessed by the DPPH method) of *T. fragrantissimus* extracts were reported to be lower than those of *T. vulgaris* extracts (Wang et al., 2014). Antioxidant capacity (DPPH method) and antimicrobial activity, against *E. coli*, of *T. fragrantissimus* aqueous extracts were recently reported and shown to be comparable to that of, *T. pulegioides* and *T. zygis* extracts (Afonso, Pereira, Valega, Silva, & Cardoso, 2018). However, the chemical composition of this thyme is largely unknown, as it is extensive radical scavenging activity and potential bioactivities. Thus the objective of this work is to obtain a phytochemical profile of *T. fragrantissimus* extracts, obtained by two distinct extraction methods, and to study their potential bioactivities, by performing several *in vitro* assays, such as anti-proliferative, anti-inflammatory, anti-oxidant and enzymatic inhibition assays, aiming to correlate its composition in bioactive molecules and *in vitro* bioactivities with potential heath-beneficial effects.

Material and methods

Standards and reagents

Methanol (HPLC or MS grade), ethanol, formic acid, acetic acid, hydrogen peroxide (30% solution), trichloroacetic acid (TCA), Folin-Ciocalteu's reagent, 2-deoxy-D-ribose, sodium nitrite, sodium nitroprusside, potassium persulfate, sodium molybdate, aluminum chloride (III), ethylenediaminetetraacetic acid (EDTA), ascorbic acid, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), thiobarbituric acid (TBA), and standards of rosmarinic acid, catechin, luteolin, apigenin, and ursolic acid were purchased from Sigma-Aldrich/Merck (Algés, Portugal). Caffeic acid was 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, Invitrogen, Portugal). Alamar Blue® was obtained from Invitrogen, Life-Technologies (Porto, Portugal).

Plant material

Aerial parts from orange-scented thyme (*Thymus fragrantissimus*) were provided from ERVITAL – Plantas Aromáticas e Medicinais (Mezio, Castro Daire, Portugal; at 40°58′47.4″ N 7°53′43.3″ W). The plants, grown under organic farming/agriculture conditions, were collected randomly from the field in July 2016. Plant identification was performed by Eng. J. Morgado (Ervital) and by the UTAD's Botanical Garden specialists. A voucher specimen was deposited in the Herbarium of UTAD's Botanical Garden. The gathered material was immediately frozen (−20 °C; 3 days), for posterior freeze-drying (FTS-System, Dura Dry TM μP, Stone Ridge, USA; −45 °C, 250 mTorr). After lyophilization, plants were reduced to a fine powder and then mixed to obtain a homogenous sample, which was stored until use (airtight container; cool, dark and dry place).

Extracts preparation

Freeze-dried orange thyme aerial portions were ground to a fine powder (using a coffee mill) and then extracted according to two extraction methods: aqueous decoction (AD) and exhaustive hydroethanolic extraction (HE), a procedure optimized to obtain all the extractable compounds within the plant material, as described in Martins-Gomes et al. (2018). In both, AD and HE extraction methods, 0.5 g of lyophilized and ground plant material were used. The HE was prepared by extracting the plant material three times with 50 mL of ethanol: water (80:20 v/v) as previously described (Martins-Gomes et al., 2018). To obtain AD extracts, the plant material (0.5 g) was extracted with distilled water (150 mL) by heating and boiling for 10 min. The suspensions were left to cool down to room temperature before filtering through a Whatman N.° 4 filter paper and then through a 0.6 μm glass fibre filter MN GF-6 under reduced pressure. Extractions were performed thrice (n = 3), and the obtained extracts were frozen and freeze dried as described earlier (Martins-Gomes et al., 2018).

Phytochemical composition (sum parameters) of extracts

The total phenolic content (TPC) was determined by the Folin–Ciocalteu method, reading the vis-absorption at 725 nm as described elsewhere (Ferreira, Silva, & Nunes, 2018; Machado, Felizardo, Fernandes-Silva, Nunes, & Barros, 2013). TPC was expressed as mg caffeic acid equivalents per gram of extract (mg CAE/g dry weight, DW). The aluminum chloride ($AlCl_3$) complex method, as described in Taghouti et al. (2020) was used for the quantification of the total flavonoids content (TFC) of extracts (complex formation was monitored at 510 nm). TFC was expressed as mg of catechin equivalents per gram of extract (mg CE/g DW). The *ortho*-diphenols content (ODC) was estimated according to the colorimetric method (absorption was read at 370 nm) based on a complex reaction with sodium molybdate dehydrate (Machado et al., 2013). Results were expressed as mg of caffeic acid equivalents per gram of extract (mg CAE/g DW).

Phenolic compounds analysis by HPLC-DAD and by HPLC-DAD-ESI/MS^n

RP-HPLC-DAD and RP-HPLC-ESI-MSn 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-MSn 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-MSn 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-MSn 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*-dihexoside and apigenin-(?)-*O*-hexuronide were quantified as apigenin; Eriodyctiol-(?)-*O*-hexoside was quantified as eriodyctiol-(7)-*O*-hexoside; luteolin-(?)-*O*-hexoside and luteolin-(?)-*O*- hexuronide were quantified as luteolin; salvianolic acid A isomer was quantified as salvianolic acid A; salvianolic acids K and I were quantified as rosmarinic acid.

Determination of oleanolic acid and ursolic acid in HE extracts

Identification and quantification of ursolic acid (UA) and oleanolic acid (OA) were performed only in HE extracts using RP-HPLC according to a previously described method (Martins-Gomes et al., 2018).

In vitro antioxidant activities

The ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) scavenging activity of the samples was determined by monitoring the rate of absorbance decay at 734 nm, as described earlier (Machado et al., 2013; Taghouti et al., 2018).

The non-site-specific hydroxyl radical (·OH) scavenging potential of the extracts was evaluated using deoxyribose test (wavelength used: 532 nm) while the site-specific (in the presence of EDTA) was performed by adding the chelating agent EDTA (1 mM), as detailed in Taghouti et al. (2018).

The nitric oxide (NO) scavenging activity was estimated by using the Griess Illosvoy reaction (monitoring at 546 nm) with modifications as described previously (Taghouti et al., 2018).

The superoxide radical ($O_2^{\bullet-}$) scavenging assay was performed as described by Tao, Zhou, Wu, and Cheng (2014), with adaptations. Briefly, to 6.7 μL of orange thyme extracts (1 mg/mL) were added 193.3 μL of a reaction solution containing: 6.43 μL of hypoxanthine (4 mM), 12.86 μL of NBT (nitro blue tetrazolium; 4 mM) and 174 μL of phosphate buffer (50 mM, pH 8). The samples were incubated at 37 °C for 2 min, and the reaction initiated by the addition of 20 μL of xanthine oxidase solution (0.04 U/mL; prepared in phosphate buffer (50 mM, pH 8) with 0.5 mM EDTA). The absorbance was measured at 570 nm (blank) and the samples were incubated for 20 min at 37 °C, followed by the addition of HCL (20 μL; 0.6 M) to end the reaction. The absorbance was measured at 570 nm at the results were expressed as % of inhibition against the control (H_2O). HE extracts were dissolved in 10% DMSO, which was previously tested and had no interference with the assay.

In vitro enzyme inhibitory activities

The inhibitory capacity of extracts on the activity of acetylcholinesterase (AChE), elastase, tyrosinase, α-amylase and α-glucosidase was evaluated using the standard assay conditions described earlier (Taghouti et al., 2018). In brief, the inhibition of AChE was determined based on Ellman's method by monitoring the hydrolysis of acetylthiocholine iodide at 25 °C spectrophotometrically at 405 nm. The inhibitory activity against elastase was measured spectrophotometrically (410 nm) at 25 °C using *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val-4-nitroanilide as substrate. For the tyrosinase inhibitory activity, the method based on the oxidation of L-DOPA at 37 °C catalyzed by tyrosinase was used (wavelength used: 475 nm). The inhibition of porcine pancreatic α-amylase activity was assayed at 37 °C based on the hydrolysis of starch. Non-degraded starch was stained by using Lugol's iodine solution and measured at 580 nm. The inhibition of α-glucosidase activity at 37 °C was tested by using 4-nitrophenyl-α-D-glucopyranoside (*p*-NPG) as a substrate. α-Glucosidase-enriched fraction from rat intestinal acetone powder was used as enzyme source, and liberated 4-nitrophenol was measured spectrophotometrically at 405 nm. Analyses were carried out in triplicate.

Anti-proliferative activity

The anti-proliferative/cytotoxic activity of orange thyme extracts was evaluated in HepG2 (human hepatoma cell line; ATCC, Rockville, USA), Caco-2 (human colon adenocarcinoma; Cell Lines Service (CLS), Eppelheim, Germany) and RAW 264.7 (mouse macrophages, Abelson murine leukemia virus-induced tumor cell line; CLS, Eppelheim, Germany) cell lines. The HepG2 and Caco-2 cells were maintained and handled according to Andreani et al. (2014) and RAW 264.7 according to Silva et al. (2020). The *in vitro* effect of AD and HE extracts on cell proliferation was determined by the Alamar Blue assay. Briefly, cells were seeded into 96-well microplates at a density of 5×10^4 cells/mL (100 μL/well) and then treated with various concentrations of extracts (0–500 μg/mL; prepared in FBS-free culture media) for 24 and 48 h. After exposure, test solutions were removed and replaced with FBS-free medium supplemented with 10% (v/v) of Alamar Blue, followed by additional 5 h incubation in 5% CO_2 at 37 °C. Cell viability was determined relative to the control cells (non-exposed cells) as described earlier (Andreani et al., 2014) by measuring the absorbance at 570 and 620 nm using a microplate reader (Multiskan EX; MTX Lab Systems, Inc., Bradenton, FL, USA). The concentrations required for inhibition of 50% of cell viability (IC_{50}) were calculated from three independent experiments (each one done in quadruplicates) (Silva et al., 2019). The extractś activity against the studied cell lines was categorized according to previously established criteria by the National Cancer Institute (NCI) guidelines (Geran, Schumacher, Abbott, Greenberg, & Macdonald, 1972): highly active $IC_{50} < 0.02$ mg/mL; moderately active IC_{50} 0.02–0.20 mg/mL; weakly active IC_{50} 0.20–0.50 mg/mL and inactive $IC_{50} > 0.50$ mg/mL.

Anti-inflammatory activity

The anti-inflammatory activity of orange thyme extracts was evaluated in RAW 264.7 cells, as described in detail in Silva et al. (2020). Briefly, RAW 264.7 cells seeded in 96-well plates ($5x10^4$ cells/mL, 100 μL/well) were incubated with non-cytotoxic concentrations of orange thyme extracts (see results) in the presence and in the absence of lipopolysaccharide (LPS; at 1 μg/mL). LPS induces nitric oxide (NO) production. After 24 h incubation, from each well, 50 μL of supernatant was transferred into a new 96-well plate, to which 50 μL/well of Griess reagent [0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride in water and 1% (w/v) sulfanilamide prepared in 5% (w/v) H_3PO_4 (v/v)] was added. After 15 min incubation (room temperature, in the dark), absorbance at 550 nm was read (Multiskan EX microplate reader; MTX Labsystems, USA). Results were quantified with resource to a standard curve performed with sodium nitrite ($NaNO_2$; in the range 0 to 100 μM) and were expressed as percentage of control (i.e. nitrite production by the control cells (LPS-stimulated cells in the absence extracts) set to 100%, that is 0% of anti-inflammatory effect.

Data analysis

For each extraction method, three individual extractions were performed, and the analyses were performed in triplicate for all the assays. The IC_{50} values for the anti-proliferative activity were calculated as described by Silva et al. (2019). Significant differences for the phenolic composition and antioxidant activity were performed using the *t*-Student test ($\alpha = 0.05$). For the comparison of the IC_{50} values, for the anti-proliferative activity and for anti-inflammatory activity, analyses of variance (ANOVA) followed by Tukey's multiple test ($\alpha = 0.05$) were performed (GraphPad Prism version 7, GraphPad Software Inc., San Diego, CA, USA).

Table 1
Extraction yields, chemical composition, and antioxidant activity of orange thyme (*T. fragrantissimus*) extracts.

		Aqueous decoction	Hydroethanolic extract	E.M.E.
Extraction yield (% *w/w*)		21.00 ± 1.54	22.92 ± 1.10	
		Chemical composition		
TPC (mg caffeic acid eq./g)	Ext.	124.26 ± 7.76	126.92 ± 13.29	
	D.P.	26.09 ± 1.63	29.09 ± 3.05	
TFC (mg catechin acid eq./g)	Ext.	167.96 ± 3.98	220.7 ± 9.42	*
	D.P.	35.27 ± 0.84	50.58 ± 2.16	*
ODP (mg caffeic acid eq./g)	Ext.	95.83 ± 3.46	126.59 ± 2.22	*
	D.P.	20.12 ± 0.73	29.01 ± 0.51	*
		Antioxidant activity		
ABTS$^{\bullet+}$ (mmol Trolox eq./g)	Ext.	0.96 ± 0.03	1.33 ± 0.08	*
	D.P.	0.20 ± 0.01	0.30 ± 0.02	*
$^{\bullet}$OH radical − EDTA (% inhibition)		25.20 ± 1.84		
$^{\bullet}$OH radical + EDTA (% inhibition)		10.12 ± 3.58		
NO$^{\bullet}$ (% inhibition)		57.72 ± 4.74		
$O_2^{\bullet-}$ (% inhibition)		48.81 ± 2.74	49.63 ± 5.42	

Abbreviations: Ext.: extract; D.P.: dry plant; E.M.E.: extraction method effect In $^{\bullet}$OH, NO$^{\bullet}$ and $O_2^{\bullet-}$ scavenging assays, the percentage of inhibition was obtained for extracts prepared at 1 mg/mL. Results are presented as mean ± standard deviation. Significant statistical differences between extraction methods (*) when ($p < 0.05$).

Results and discussion

Extract yield and chemical composition (sum parameters) of extracts

In this work, two extraction methods were used to obtain the orange thyme (*T. frangatissimus*) extracts: 1) an exhaustive hydroethanolic (HE) extraction intended to obtain the total "free" phenolic compounds present in the plant in order to study its phenolic composition (Martins-Gomes et al., 2018); 2) an aqueous decoction (AD) aiming to mimic the common procedure of beverage preparation for human consumption. This last procedure allows analyzing the phenolic compounds that will be potentially available when these plants are used as herbal teas and as condiments.

Extraction yields and phytochemical characterization of the AD and HE *T. fragrantissimus* extracts by using sum parameters are presented in Table 1. The extraction yields obtained for the two extraction methods used were similar (Table 1). Concerning the AD extract, the yield obtained in this work (21.00 ± 1.54 %, Table 1) is higher than that described by Afonso et al. (2018), who reported an extraction yield of 16%. Nevertheless, the extraction conditions were significantly different (5 g plant per 100 mL of water, for 15 min) from that used in this work. However, AD extract yield (Table 1) is identical to that of *T. vulgaris* (Taghouti et al., 2020), but higher than other thyme species, such as *Thymus pulegioi*des (Taghouti et al., 2018).

The HE extraction yield obtained for *T. fragrantissimus* is in line with that obtained for *T. zygis* subsp. *zygis* (26.0% (Silva et al., 2020)), *T. carnosus* (24.7% (Martins-Gomes et al., 2018)), *T. vulgaris* (24.3% (Taghouti et al., 2020)), *T. pulegioides* (22.2% (Taghouti et al., 2018)), grown at the same location and using the same extraction procedure, although being higher than that obtained for *T. citriodorus* (14.1% (Taghouti et al., 2020)) and *T. mastichina* (13.8% (Taghouti et al., 2020)). These difference might result from a species effect or from the time of year in which they were harvested, as *T. fragrantissimus* was harvested at the end of July (end of blooming stage) and the other ones in October (post-blooming, end fructification stage), the latter hypothesis still needs to be confirmed with more experimental data and other *Thymus* species harvested in the same place at both stages.

The total phenolic compounds (TPC) extracted with HE method were not significantly different from the AD extraction (Table 1). The orange thyme TPC value obtained for the AD extraction procedure presented in this work is slightly higher than that obtained by Afonso et al. (2018) (327 vs. 287 mg GAE/g extract).

Comparing the TPC content *per* gram of dry plant, in plants collected in the same place and extracted with the same HE extraction method, we observed an order for TPC contents (in mg CA eq./g D.P.), *T. pulegioides* (56.1 (Taghouti et al., 2018)) > *T. zygis* subsp. *zygis* (42.7, (Silva et al., 2020)) ∼ *T. carnosus* (41.9 (Martins-Gomes et al., 2018))≫ *T. fragrantissimus* (29.1; Table 1) ∼ *Thymus citriodorus* (27.7 (Taghouti et al., 2020)) ∼ *T. vulgaris* (25.12 (Taghouti et al., 2020)) ∼ *T. mastichina* (24.6 (Taghouti et al., 2020)). Concerning the AD extraction, *T. fragrantissimus* AD extracts allowed to recover TPC similar to *T. zygis* subsp. *zygis* (29.1 (Silva et al., 2020)) and *T. pulegioides* (26.1 (Taghouti et al., 2018)), higher than *T. vulgaris* (21.6 (Taghouti et al., 2020)), *T. citriodorus* (15.5 (Taghouti et al., 2020)) and *T. mastichina* (12.5 (Taghouti et al., 2020)) but lower than *T. carnosus* (35.7 (Martins-Gomes et al., 2018)).

The amount of TFC extracted by HE extraction for *T. fragrantissimus* was significantly higher than those obtained by AD extraction (Table 1), the same being observed for the ODP (Table 1). Altogether these data highlight the value of *T. fragrantissimus* as a thyme species with high content in potential bioactive molecules similar to the highly used species *T. vulgaris* (used worldwide as condiment, either in fresh or dried), *T. citriodorus* and *T. mastichina* (the later used as herbal teas and also as condiments).

Profile of phytochemical compounds

The phenolic composition of *T. fragrantissimus* HE and AD extracts was determined by HPLC-DAD and HPLC-MS^n, aiming to have a deeper understanding of the *T. fragrantissimus* chemical composition and the relation with its extract's bioactivities. The HE and AD extracts phenolic profiles, as well as their concentrations, are shown in Fig. 1 and in Table 2. As observed, the relative amount of phenolic compounds determined by HPLC-DAD is consistent with the obtained TFC and OPD contents (Table 1), nevertheless they are not consistent with the TPC results (Table 1). This can be due to the presence of non-phenolic compounds in the AD extract of *T. fragrantissimus* that can react with the Folin-Ciocaulteu reagent, like reducing sugars (Rover & Brown, 2013)

As shown in Table 2, rosmarinic acid was the most abundant phenolic compound in *T. fragrantissimus* HE extracts (50% of the total phenolic compounds extracted by HE) but significant amounts of luteolin-(?)-*O*-hexuronide were also present (28% of the total phenolic compounds of HE, Table 2). Rosmarinic acid is commonly found in high amounts in most *Thymus* species, such is the case of *T. fragrantissiums* that also characterized by high contents of rosmarinic acid (Table 2), having in account that exhaustive HE extraction reflects the phenolic content of the plant (Martins-Gomes et al., 2018). High contents of rosmarinic acid were also detected in HE extracts of other *Thymus* species (as % of total phenolic acids), such as *T. vulgaris* and *T. citriodorus* (70% and 51%, respectively (Taghouti et al., 2020)). Contrasting with other species, such as in *T. carnosus*, that contains relative low amounts of rosmarinic acid (17% (Martins-Gomes et al., 2018)). Nevertheless, this *Thymus* also contains significant amounts of flavonoids (39% of the total phenolic compounds in HE extracts), especially luteolin-(?)-*O*-hexuronide (72% of the total extractable flavonoids in HE extract), as mentioned above. *T. fragrantissimus* is the *Thymus* species studied by our group that contains the third highest levels of flavonoids quantified in the HE extracts [(*T. pulegioides* (61%), *T. zygis* subsp. *zygis* (42%),

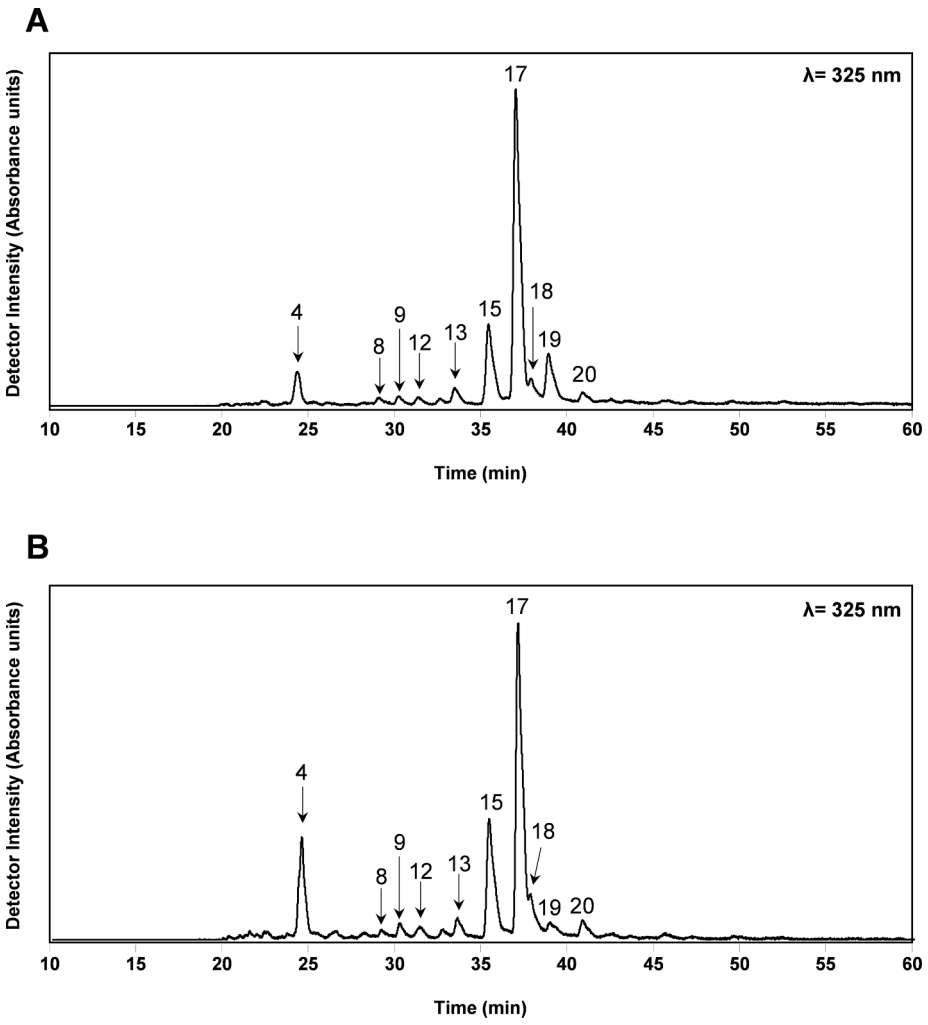


Fig. 1. Chromatograms of (A) hydroethanolic (HE) and (B) aqueous decoction (AD) extracts obtained from orange thyme (*T. fragrantissimus*). For peak identification please refer to Table 2.

T. mastichina (39%), *T. citrodorus* (24%), *T. vulgaris* (16%), and *T. carnosus* (6%)].

On the other hand, luteolin-(?)-*O*-hexuronide was the most abundant phenolic compound present in orange thyme AD extract (42% of the total phenolic compounds extracted by AD), followed by rosmarinic acid that was present in lower but still significant amounts (35% of the total phenolic compounds extracted by AD). Interestingly, AD extract contained significant amounts of eriodictyol-(?)-*O*-hexuronide (14% of the total phenolic compounds extracted by AD), a quantity identical to that in the HE extract (Table 2; 7% of the total phenolic compounds extracted by HE), indicating that this compound is easily extracted. By the other hand, the amount of rosmarinic acid extracted by AD method represents only 37% of the rosmarinic acid extracted by HE, but for luteolin-(?)-*O*-hexuronide and eriodictyol-(?)-*O*-hexuronide the amount extracted by AD represents 76% and 100% of that extracted by HE. This can be due to the hexuronic acid group present in these phenolic compounds that confer a negative charge and probably more solubility.

The most abundant phenolic compounds described for *T. fragrantissimus* AD extracts (Table 2) are in agreement with those described by Afonso et al. (2018) for aqueous extracts of *T. fragrantissimus* (harvested in the same location), although the relative amounts found were different, with rosmarinic acid accounting for 64% and for luteolin-(?)-*O*-hexuronide for 13% of the extracted phenolic compounds, which might have resulted from extraction procedure (different from the one in current work) or from harvesting period.

A close comparison of the relative phenolic composition of HE extracts from *T. fragrantissimus* (Table 2) and *T. vulgaris* (Taghouti et al., 2020) show both similarities and differences between the two *Thymus*. Both *Thymus* contain as the main phenolic compound rosmarinic acid, followed by luteolin-(?)-*O*-hexuronide and smaller amounts of salvianolic acid K and luteolin-(?)-*O*-hexoside. *T. fragrantissimus* HE presented low amounts (below quantification limit) of apigenin-(6,8)-*C*-diglucoside, eriodictyol-(?)-*O*-hexoside. *T. vulgaris* HE extracts presented significantly higher amounts of salvianolic acid I than *T. fragrantissimus*, but on the other hand *T. vulgaris* didn't contain eriodictyol-(?)-*O*-hexuronide but this flavonoid was present in significant amounts in the HE extract of *T. fragrantissimus* (Table 2). Other common feature between *T. fragrantissimus* and *T. vulgaris* is that the triterpenoids ursolic and oleanolic acids were not detected in the HE extracts of both thyme plants.

In vitro antioxidant activities

T. fragrantissimus HE extracts (at 1 mg/mL) showed higher $ABTS^{+\bullet}$ radical scavenging activity (~0.30 Trolox eq./g dry plant, Table 1) than the AD extracts (~0.20 mmol Trolox eq./g dry plant, Table 1). $ABTS^{+\bullet}$ radical scavenging activity obtained for HE extracts (Table 1) was lower than that of *T. pulegioides* HE extracts (0.34 mmol Trolox eq./g D.P.; (Taghouti et al., 2018) but higher than that of *T. zygis* subsp. *zygis* (0.25 mmol Trolox eq./g D.P.; (Silva et al., 2020)), *T. vulgaris* and T. *citriodorus*

Table 2
Phytochemical composition of hydroethanolic (HE) and aqueous decoction (AD) extracts of orange thyme (*T. fragrantissimus*) as determined by HPLC/DAD-ESI/MS^n.

	Compound	R.T.	ESI-MS2	Quantification (mg/g of extract)				E.M.E.	
				AD		HE		Ext.	D.P.
				Ext.	D.P.	Ext.	D.P.		
1	Unknown	20.28 ± 0.01	[465]:327;303;285;176	n.q.	n.q.	n.q.	n.q.		
2	Eriodictyol-di-(?)-*O*-hexoside	22.61 ± 0.04	[611];287	n.q.	n.q.	n.q.	n.q.		
3	Hydroxyjasmonic acid–(?)-*O*-hexoside	24.58 ± 0.06	[387]:369;225;207;163	n.q.	n.q.	n.q.	n.q.		
4	Apigenin-(6,8)-*C*-dihexoside	24.38 ± 0.01	[593]:575;503;473;383;353	n.q.	n.q.	n.q.	n.q.		
5	Eriodictyol-(?)-*O*-hexoside-deoxyhexoside	28.28 ± 0.10	[595]:287	n.q.	n.q.	n.q.	n.q.		
6	Eriodictyol-(?)-*O*-hexoside	28.72 ± 0.05	[449]:287	n.q.	n.q.	n.q.	n.q.		
7	Naringenin-*O*-hexoside	29.07 ± 0.80	[433]:313;271	n.q.	n.q.	n.q.	n.q.		
8	Quercetin-(?)-*O*-hexoside	29.15 ± 0.01	[463]:301	n.q.	n.q.	n.q.	n.q.		
9	Eriodictyol-(?)-*O*-hexuronide	30.26 ± 0.05	[463]:287;175	10.10 ± 0.69	2.12 ± 0.14	9.12 ± 0.59	2.09 ± 0.14		
10	Quercetin-(?)-*O*-hexuronide	31.16 ± 0.05	[477]:301	n.q.	n.q.	n.q.	n.q.		
11	Luteolin-(?)-*O*-hexoside	31.48 ± 0.05	[447]:285	1.03 ± 0.17	0.22 ± 0.03	1.23 ± 0.24	0.28 ± 0.05		
12	Sagerinic acid isomer	31.37 ± 0.06	[719]:539;521;495;477;411;359	n.q.	n.q.	n.q.	n.q.		
13	Luteolin-(?)-*O*-hexoside	33.54 ± 0.05	[447]:285	1.35 ± 0.20	0.28 ± 0.04	1.24 ± 0.07	0.29 ± 0.02		
14	Naringenin-*O*-hexoside	33.73 ± 0.05	[433]:313;271	n.q.	n.q.	n.q.	n.q.		
15	Luteolin-(?)-*O*-hexuronide	35.47 ± 0.10	[461]:285;175	29.50 ± 2.42	6.19 ± 0.51	35.57 ± 2.99	8.15 ± 0.67		*
16	Chrysoeriol-(?)-*O*-hexoside	36.67 ± 0.01	[461]:299	n.q.	n.q.	n.q.	n.q.		
17	Rosmarinic acid	37.10 ± 0.01	[359]:223;197;179;161	25.11 ± 2.82	5.27 ± 0.59	62.30 ± 1.37	14.28 ± 0.31	*	*
18	Salvianolic acid K	37.89 ± 0.13	[555]:537;493;359	3.07 ± 0.29	0.64 ± 0.06	4.13 ± 0.15	0.95 ± 0.03	*	*
19	Salvianolic acid I	39.00 ± 0.20	[537]:493;448;359;339;313	0.26 ± 0.10	0.05 ± 0.01	9.77 ± 0.08	2.24 ± 0.02	*	*
20	Apigenin-(?)-*O*-hexuronide	41.00 ± 0.06	[445]:269;175	0.98 ± 0.25	0.20 ± 0.05	1.97 ± 0.23	0.45 ± 0.05	*	*
21	Quercetin-(?)-*O*-hexoside-hexuronide	42.01 ± 0.07	[639]:301	n.q.	n.q.	n.q.	n.q.		
			Total phenolic compounds	71.41 ± 5.85	14.9 ± 1.23	125.32 ± 3.78	28.72 ± 0.87	*	*
			Total flavonoids	42.97 ± 3.37	9.02 ± 0.71	49.12 ± 2.78	11.26 ± 0.64		*
			Total phenolic acids	28.44 ± 3.16	5.97 ± 0.66	76.19 ± 1.53	17.46 ± 0.35	*	*

Abbreviations: AD: aqueous decoction; HE: hydroethanolic extractions; RT: retention time; ESI-MS2-Fragment ions obtained after fragmentation of the pseudo-molecular ion [M]; n.q.: not quantified (but detected); E.M.E.: extraction method effect; (*) denotes significant statistical differences (*t*-Student) between extraction methods, if ($p < 0.05$). Results, from n = 3 different extractions, per extract, are presented as mean ± standard deviation.

(0.22 mmol Trolox eq./g D.P.; (Taghouti et al., 2020)). In contrast, ABTS$^{\bullet+}$ radical scavenging activity of *T. fragrantissimus* AD extracts (0.20 mmol Trolox eq./g D.P.; Table 1) was similar to that found for *T. vulgaris* (0.20 mmol Trolox eq./g dry plant; (Taghouti et al., 2020)) and *T. zygis* subsp. *zygis* (0.23 mmol Trolox eq./g DP (Silva et al., 2020)) but higher than that described for the other *Thymus* species, such as T. *pulegioides* (0.15 mmol Trolox eq./g D.P. (Taghouti et al., 2018) and *T. mastichina* 0.08 mmol Trolox eq./g D.P. (Taghouti et al., 2020).

T. fragrantissimus AD extract (at 1 mg/mL) exhibited low non-site-specific inhibition activity and site-specific inhibition activity (Table 1), presenting a lower inhibition capacity against hydroxyl radical (·OH) compared to other *Thymus*, such as *T. carnosus* (41% (Martins-Gomes et al., 2018)), and *T. citriodorus* (38% (Taghouti et al., 2020)). Both, non-site-specific inhibition activity and site-specific inhibition, obtained for orange thyme are identical to that of *T. vulgaris* AD extracts (Taghouti et al., 2020). Concerning the scavenging of the NO radical *T. fragantissimus* also showed a similar inhibition percentage to that of *T. vulgaris* (58%; (Taghouti et al., 2020)) and higher than that described for other *Thymus* (e.g. *T. carnosus* (42%; (Martins-Gomes et al., 2018)), On the other hand the superoxide scavenging activity of both HE and AD extracts were similar (Table 1; $p > 0.05$) and are identical to those reported to *T. vulgaris* water extracts (~45% inhibition (Kim, Yang, Lee, & Kang, 2011)) and slightly higher than those of *T. zygis* methanolic extracts (~40% (Soares, Dinis, Cunha, & Almeida, 1997). These data highlight the high antioxidant capacity of orange thyme extracts.

In vitro enzyme inhibitory activities

The phenolic-rich extracts from orange thyme were tested for their inhibitory ability on the activity of acetylcholinesterase (AChE), elastase, tyrosinase, α-amylase and α-glucosidase. The inhibition percentage

Table 3
In vitro assessment of orange thyme (*T. fragrantissimus*) extracts' inhibitory activity against acetylcholinesterase (AChE), tyrosinase, elastase, α-glucosidade and α-amylase.

	Enzymatic inhibition (% inhibition)				
	AChE	Tyrosinase	Elastase	α-Amylase	α-Glucosidase
AD	12.1 ± 6.65	56.30 ± 1.9	n.d.	9.30 ± 2.43	12.67 ± 2.10
HE	27.30 ± 0.99	42.40 ± 4.01	48.69 ± 3.35	10.24 ± 0.37	11.41 ± 1.96

Results are presented as mean ± S.D. (n = 3), for extracts prepared at 0.5 mg/mL; n.d., not detected.

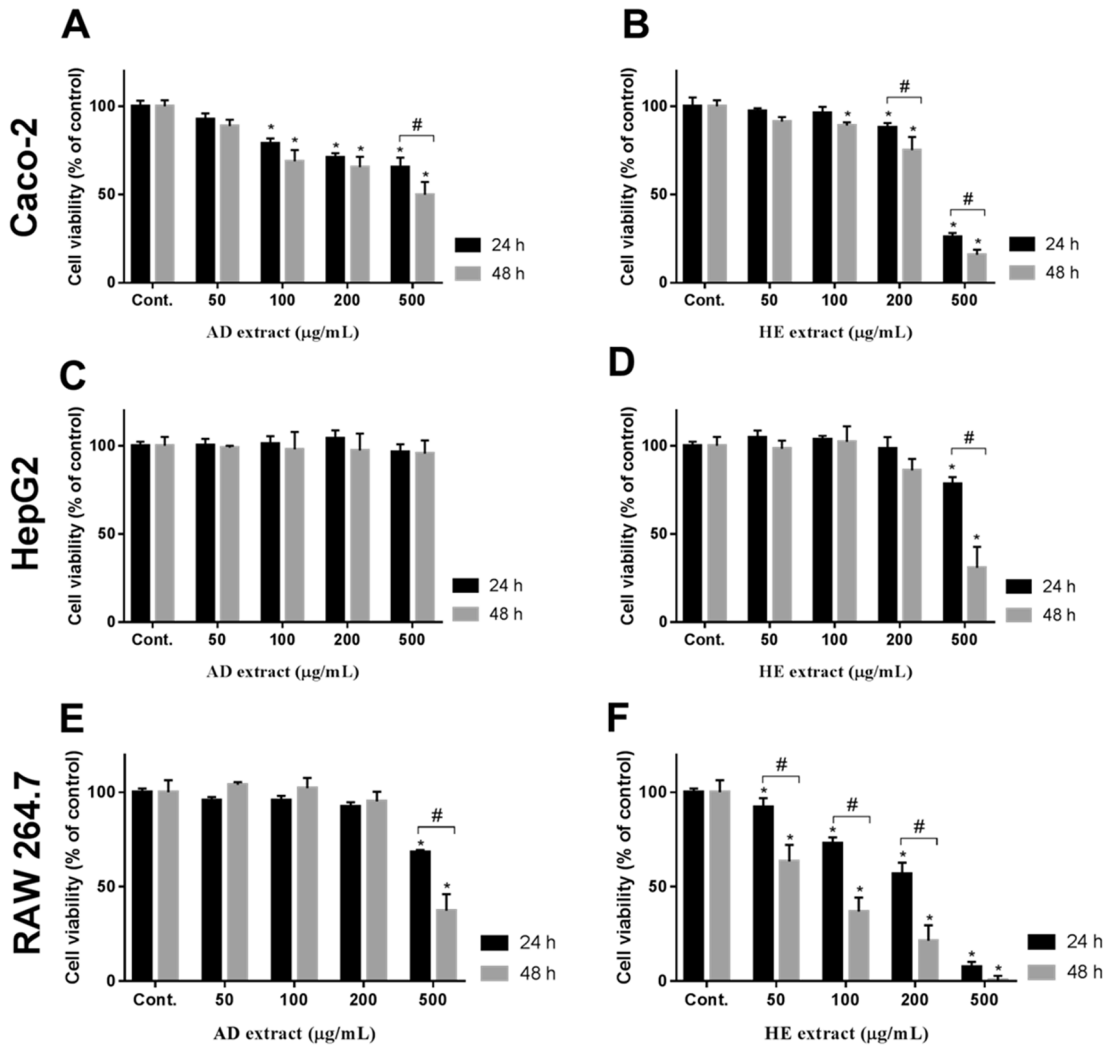
of the orange thyme extracts (at 0.5 mg/mL) are presented in Table 3. The HE extract presented a significantly higher inhibition of the AChE activity than AD extract. However, the AChE inhibition values obtained for orange thyme are lower than that reported for *T. pulegeoides* (82% and 89% for AD and HE extracts respectively; (Taghouti et al., 2018)). *Thymus vulgaris* ethanolic extracts (Vladimir-Knezevic et al., 2014) as well as ethanolic and aqueous extracts of *Thymus serpyllum* (Mata et al., 2007) exhibited similar AChE inhibiting effects as the here reported to orange thyme extracts. Overall, these results have been attributed to the presence of rosmarinic acid in the extracts although several herbal extracts containing luteolin glycosides also tend to possess acetylcholinesterase inhibitory activity (Choi et al., 2014). Both extracts presented a significant inhibitory effect of tyrosinase activity. However, lower inhibitory activities were observed for orange thyme HE extract against tyrosinase (~42%, at 0.5 mg/mL; Table 3) in comparison with the same amount of *T. pulegioides* HE extracts (above 90% inhibition, (Taghouti et al., 2018)). Up to now, there have been no other reports in the literature on the tyrosinase-inhibitory activity of plants from this genus although these data show that *Thymus* extracts are effective against this enzyme. When individual compounds are considered, rosmarinic acid has been shown to display a strong tyrosinase inhibitory activity (Oliveira, Palu, Weffort-Santos, & Oliveira, 2013). However, other compounds such as flavonoid derivatives (Orhan & Khan, 2014) may also be responsible for the results observed. Beyond being implicated in melanin synthesis in skin and hair, tyrosinase also contributes to the formation of neuromelanin in human brain that, by interacting with many toxicants (such as metals and pesticides), may play a significant role in both initiation and progression of neurodegeneration (Karlsson & Lindquist, 2016). This may indicate that orange thyme has potential health benefits at the neuroprotection level.

Contrarily to what has been described for the extracts obtained from other *Thymus* species, such as *T. pulegioides* (Taghouti et al., 2018), where the HE extract was active against elastase whereas aqueous extracts showed low activity, for *T. fragrantissimus* we found that 0.5 mg/mL of HE extract inhibited about 50% of elastase activity while AD extract did not show inhibitory activity (Table 3). Results of α-amylase and α-glucosidase inhibition activities (Table 3) showed that both extracts (at 0.5 mg/mL) have low ability to inhibit these enzymes (about 10% inhibition is observed). This means that extracts from orange thyme have poor anti-diabetic activity, as seen by the low inhibition capacity of both α-amylase and α-glucosidase. Comparable data were previously reported for *T. vulgaris* alcoholic extracts (Aljarah & Hameed, 2018) as well as other *Thymus* species (Taghouti et al., 2018). However, the observed inhibition capacity described in Table 3, might be an important indicator that the content of these extracts in bioactive molecules has a potential health benefit, in several aspects, such as in neuroprotection (as inhibitors of acetylcholinesterase are candidates to treat neurodegenerative diseases) and "anti-aging" (as inhibitors of elastase are used in anti-wrinkle cosmetics). In addition, the tyrosinase inhibitory potential is also of interest in skin health research, cosmetics and agricultural industries, but also in food industry.

Anti-proliferative activity

Anti-proliferative/cytotoxic activity of orange thyme extracts was assessed in three cell lines, Caco-2, HepG2 and RAW 264.7 cells, and results are shown in Fig. 2. As observed in Fig. 2, in general the cell's exposure to the extracts causes a dose-dependent reduction on cell viability, with exception of HepG2 cells incubated with AD extract (Fig. 2C) which seems unaffected by this extract concentration (at 500 μg/mL cell viability is ~ 100% of control). Generally, HE extract had a higher effect on cell viability than aqueous extracts, and, Raw 264.7 cells (Fig. 2F) are the most affected by HE extracts while HepG2 cells (Fig. 2D) are the least affected, at both exposure times. Comparing the IC_{50} values (Fig. 2 2G) obtained for HepG2 cells after 24 h and 48 h exposure to HE extract reveals a time-dependent effect, although at both incubation times the extract presents a week activity at this cell line. Concerning the extracts' effect at Caco-2 cells (Fig. 2A and B), AD induces a dose-dependent effect although the time-dependent effect is week and IC_{50} values at both exposure times are higher than 500 μg/mL (considered inactive according to Geran et al. (1972)). However, HE extract is more effective at both exposure times, but considered weakly active as IC_{50} are between 0.20 and 0.50 mg/mL (Fig. 2G). As observed in Fig. 2, the cell viability of the murine macrophage cell line (Raw 264.7) is the most affected by orange thyme extracts, although the calculated IC_{50}'s (Fig. 2G) indicate that the HE extract is considered moderately active while AD extract is weakly active at 48 h exposure but considered inactive at 24 h exposure, according to Geran et al. (1972).

Over the years many studies have revealed anti-proliferative properties of phenolic compounds in different tumor and non-tumor cell lines although the underlying mechanisms are still not fully understood. In this study, it was demonstrated that orange thyme extracts are capable of eliciting an anti-proliferative action, in a dose- and time-dependent manner with the effect also being dependent on the tested cell line (Fig. 2). Nevertheless, the responses were not linear and only weakly related to the solvent extraction method suggesting a synergistic effect between several compounds present in both extracts. To the best of our knowledge, no previous studies have assessed the effect of orange thyme extracts against tumor and non-tumor cell lines. Still, the effects observed may be comparable to those of *T. vulgaris* extracts. In fact, HE extract of *T. vulgaris* was shown to decrease cell proliferation in a dose-dependent manner in HepG2 cells (Kozics et al., 2013; Taghouti et al., 2020), but with IC_{50} values higher than 0.5 mg/mL. Also, in several tumor cell lines (HeLa, Ag8.653, SK-Br-3, K562, MDA-MB-435 and U937) (Amirghofran & Karimi, 2001) and in other cancer-derived cell lines (THP-1 and PBMC) (Ayesh, Abed, & Faris, 2014) *T. vulgaris* decreased cell proliferation although showing slight deviations in the IC_{50} values compared to those determined here (Fig. 2G). In addition, thyme extracts obtained from *T. pulegioides* and *T. carnosus* Boiss showed similar anti-proliferative activity against Caco-2 and HepG2 cells (Martins-Gomes et al., 2018; Taghouti et al., 2018). In general, the reported effects could be associated to the natural variability inherent to different plant species and may be further induced by different edaphoclimatic conditions that influence their secondary metabolism (Ramakrishna & Ravishankar, 2011). Also, interactions between compounds in solution cannot be excluded resulting in potential synergistic or antagonistic effects. A previous study analyzed potential cytotoxic and anti-proliferative capacities of different extracts obtained from plants belonging to the *Lamiaceae* family and compared the effects to those of individual phenolic constituents (Berdowska et al., 2013). Although isolated compounds were demonstrated to be cytotoxic, too, more beneficial properties were obtained when the extracts were applied, comprising their complex mixtures (Berdowska et al., 2013). Having in account the IC_{50} values here reported for the effect of orange thyme extracts on Caco-2 and HepG2 (Fig. 2G), which are relatively high, we may conclude that orange thyme is not cytotoxic when used in regular doses in food preparations (as condiment or other), and due to its orange-scented aroma it is a good alternative to common thyme.



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		IC_{50} (μg/mL)		Exposure time effect		Extraction method effect
		AD	HE	AD	HE	
Caco-2	24h	> 1000	362.2 ± 8.5	*	*	*
	48h	470.5 ± 73.1	284.0 ± 12.7			*
HepG2	24h	> 1000	708.6 ± 80.1		*	*
	48h	> 1000	380.2 ± 19.9			*
Raw 264.7	24h	774.4 ± 49.8	196.8 ± 10.4	*	*	*
	48h	437.9 ± 12.4	72.1 ± 4.9			*

Fig. 2. Effect of orange thyme (*T. fragrantissimus*) aqueous decoction (AD) and hydroethanolic (HE) extracts on Caco-2 (**A** and **B**), HepG2 (**C** and **D**) and Raw 264.7 (**E** and **F**) cells, after 24 h or 48 h exposure, as indicated. (**G**) Values of IC_{50} (half maximal inhibitory concentration) obtained for Caco-2, HepG2 and Raw 264.7 cells exposed orange thyme AD and HE extracts, as denoted. Results are expressed as mean ± SD (n = 4). Statistically significant differences ($p < 0.05$) between the control and sample concentrations at respective incubation time are denoted by an *, and those between exposure times, at the same concentration, are denoted by a #.

Anti-inflammatory activity

The anti-inflammatory activity of orange thyme (*T. fragrantissimus*) was evaluated on RAW 264.7 cells (a macrophage cell line), as these cells have the ability to release nitric oxide (NO) when exposed to bacterial lipopolysaccharides (LPS)-induced as a result of inflammatory signaling activation that culminates with the enhancement of nitric oxide synthase activity. Thus, the anti-inflammatory action of AD and HE extracts was assessed by their capacity to reduce the amount of NO released by LPS-stimulated macrophages. Taking into account the cell viability results for RAW 264.7 cells (Fig. 2E, F and G), non-cytotoxic concentrations of orange thyme extracts were selected to perform the

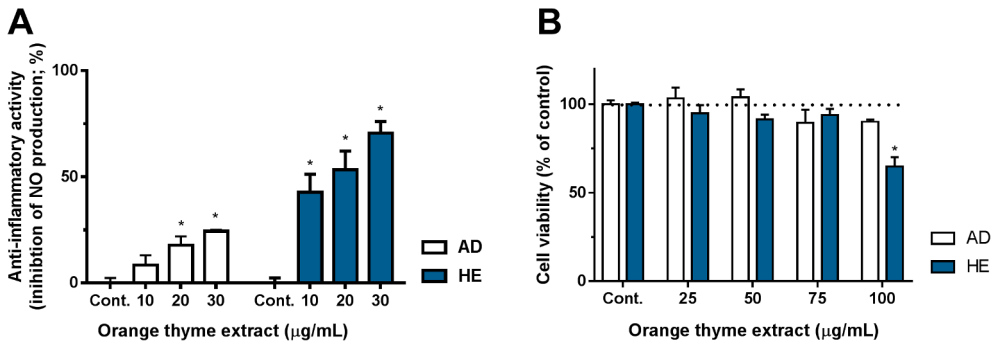

Fig. 3. Anti-inflammatory activity of orange thyme (*T. fragrantissimus*) extracts. (A) Inhibition of nitric oxide (NO) release by LPS-stimulated RAW 264.7 cells when induced by AD (left bars, white) and by HE (right bars, blue) extracts, expressed as percentage of control (see methods for details). (B) RAW 264.7 cells viability upon exposure to AD (white bars) and HE (blue bars) extracts (see methods for details). Results are expressed as mean ± SD (n = 4 independent assays).

anti-inflammatory assay, as NO syntheses is dependent on cell viability. Fig. 3B shows that exposure of RAW 264.7 cells to AD and HE extracts, for 24 h, is not cytotoxic for concentrations up to 75 μg/mL ($p > 0.05$, in relation to control), however 50 μg/mL of HE slightly decreases cell viability (91.5 ± 2.7% of control; $p > 0.05$), and thus concentrations up to 30 μg/mL of both extracts were selected to perform the anti-inflammatory assay (Fig. 3A). As observed (Fig. 3A), both extracts produced a dose-dependent inhibition of NO release from LPS-stimulated RAW 264.7 cells, which is indicate of anti-inflammatory activity. To note that, at 10 μg/mL the HE extract produces an about 5-fold higher effect, comparing with AD extract, and at 20 and 30 μg/mL and about 3-fold effect is observed (Fig. 3B), which in part might result from a higher content in phenolic compounds in HE extract compared to AD (Table 1 and 2). Data in Table 2 show that HE extract has a 3-fold higher content in phenolic acids, which also reflects the anti-inflammatory activity being these compounds good candidates to further studies using isolated molecules. Indeed, some phenolic acids have been reported to induce anti-inflammatory activity, *in vitro* and *in vivo*, as is the case of rosmarinic acid, salvianolic acids and their derivatives (Afonso, Pereira, & Cardoso, 2020; Jiang et al., 2018; Martins-Gomes et al., 2018; Thammason, Khetkam, Pabuprapap, Suksamrarn, & Kunthalert, 2018; Wei et al., 2018), as well as some flavonoids common in *Thymus* species, and present in orange thyme, such as luteolin and apigenin and their derivatives (Afonso et al., 2020; Xia et al., 2016).

Conclusion

Orange thyme (*T. fragrantissimus*) extracts showed high content in rosmarinic acid and in luteolin-(?)-*O*-hexuronide, and considerable amounts in salvianolic acids. Both orange thyme extracts presented high antioxidant capacity, namely as nitric oxide and as radical superoxide scavengers. A moderate anti-acetylcholinesterase activity and high anti–tyrosinase activity may suggest a potential health benefit at neuroprotection level. Potential effect as anti-aging is also suggested by the moderate anti-elastase activity. Moreover, the anti-inflammatory activity of the extracts associated with the weak anti-proliferative/cytotoxic activity against the tested cell lines supports its safe use as food and can reinforce its use as a functional food with several beneficial health effects. It can be concluded that the most bioactive compounds can be extracted using both ethanol and water demonstrating the potential of these extracts as future sources of novel antioxidants to be used in different food and pharmaceutical formulations. However, the isolation and identification of the molecule(s) that are responsible for the biological activities detected are required to clarify the mechanisms underlying the beneficial health effects observed in this work.

CRediT authorship contribution statement

Amélia M. Silva: Conceptualization, Investigation, Formal analysis, Data curation, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **Luís M. Félix:** Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Isabel Teixeira:** Investigation, Writing – review & editing. **Carlos Martins-Gomes:** Investigation, Writing – review & editing. **Judith Schäfer:** Investigation, Writing – review & editing. **Eliana B. Souto:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Mirko Bunzel:** Investigation, Formal analysis, Data curation, Funding acquisition, Resources, Writing – review & editing. Fernando M. Nunes: Conceptualization, Investigation, Formal analysis, Funding acquisition, Resources, 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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