

**Decoction, infusion and hydroalcoholic extract of cultivated thyme:
Antioxidant and antibacterial activities, and phenolic characterization**

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Running Title: Antioxidant/antibacterial activity and phenolic compounds of thyme

Abstract

Bioactivity of thyme has been described, but mostly related to its essential oils, while studies with aqueous extracts are scarce. Herein, the antioxidant and antibacterial properties of decoction, infusion and hydroalcoholic extract, as also their phenolic compounds, were evaluated and compared. Decoction showed the highest concentration of phenolic compounds (either phenolic acids or flavonoids), followed by infusion and hydroalcoholic extract. In general, the samples were effective against gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and gram-negative (*Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Enterococcus aerogenes*, *Proteus vulgaris* and *Enterobacter sakazakii*) bacteria, with decoction presenting the most pronounced effect. This sample also displayed the highest radical scavenging activity and reducing power. Data obtained support the idea that compounds with strong antioxidant and antibacterial activities are also water-soluble. Furthermore, the use of thyme infusion and decoction, by both internal and external use, at recommended doses, is safe and no adverse reactions have been described.

Keywords: *Thymus vulgaris* L.; decoction/infusion; hydroalcoholic extract; phenolic compounds; antioxidant/antibacterial activity

1. Introduction

Continuous exposure to various sources of aggressors leads to an increase in the production of reactive species, cellular deterioration and, in long term, contributes to aging and other organic disorders. Oxygen, nitrogen and sulphur reactive species, including free radicals, have been implicated in the onset of various diseases such as neurodegenerative diseases, cancer, cardiovascular diseases, cataracts, rheumatism, ulcers, and atherosclerosis. Antioxidants have the ability to neutralize these species, protecting cell's molecules namely, proteins, lipids, carbohydrates and nucleic acids (Carocho & Ferreira, 2013; Chaturvedi & Beal, 2013; Rubi3, Motilva, & Romero, 2013). Furthermore, since the middle of twentieth century, a continuous and significant increase in the incidence and prevalence of opportunistic infections has been observed, not only in immunocompromised and hospitalized patients, but also in the general population. The indiscriminate and disseminate use of antimicrobial agents has contributed to an increase of microbiological resistance (Van Vuuren, Suliman, & Viljoen, 2009; Kon & Rai, 2012).

The use of medicinal plants for prevention or treatment of various conditions is an old tradition, but recently, has sparked an increasing interest by researchers that have been studying their biological properties (Silva & Fernandes J3nior, 2010; Rubi3, Motilva, & Romero, 2013). In fact, natural matrices can give an exogenous contribution to the detoxification and elimination of reactive species (Carocho & Ferreira, 2013) and also to the discovery of novel antimicrobial compounds (Maroyi, 2013).

Thymus vulgaris L., commonly known as thyme, could be one of those matrices, providing a wide range of biomolecules, such as phenolic compounds. Thyme is a perennial subshrub widely cultivated around the world and belongs to the Lamiaceae (lipped) family. It is used, since ancient times, both for nutritional and medicinal

purposes. Commonly known by its multiple health benefits, thyme infusions, decoctions or essential oils are mostly used due to antispasmodic activity in smooth muscle (bronchial and intestinal), immunostimulant, expectorant, mucolytic, antitussive/bronchodilator, and digestive/eupeptic properties (Vanaclocha & Cañigüeral, 2003). Some studies have also reported its antioxidant (Kulisic, Radonic, & Milos, 2005; Viuda-Martos, Navajas, Zapata, Fernández-López, & Pérez-Álvarez, 2010) and antimicrobial (Imelouane et al., 2009; Kon & Rai, 2012) properties, but mostly referring to its essential oils, whereas only a few studies report the bioactivities of aqueous extracts, namely decoctions or infusions (Kulisic, Dragovi-Uzelac, & Milos, 2006; Roby, Sarhan, Selim, & Khalel, 2013). Regarding chemical composition, the majority of the studies also refer to essential oils (e.g., Giordani et al., 2004; Kulisic, Radonic, & Milos, 2005; Viuda-Martos, Ruíz-Navajas, Fernández-López, & Pérez-Álvarez, 2007; Imelouane et al., 2009), while less work has been done in relation to its phenolic composition (Dapkevicius et al., 2002; Boros et al., 2010; Miron et al., 2011; Nagy et al., 2011; Koşar, Dorman, & Hiltunen, 2005; Vergara-Salinas et al., 2012; Roby et al., 2013). The main phenolic compounds identified in the studied thyme samples were rosmarinic acid, caffeic and ferulic acids and their derivatives. Furthermore, Kulisic, Dragovi-Uzelac and Milos (2006) studied the antioxidant activity of thyme infusions as related to the phenolic composition, but as far as we know no reports are available in decoctions.

In the present work, the antioxidant and antibacterial properties of decoction, infusion and hydroalcoholic extracts of thyme (*Thymus vulgaris* L.) were evaluated, compared and characterized in terms of their composition of phenolic compounds.

2. Materials and methods

2.1. Sample

Flowering aerial parts (leaves and flowers, separated from branches) of *Thymus vulgaris* L., previously dried, supplied by Soria Natural (Garray - Soria, Spain), were obtained in September 2012. The sample was a clean product, with monitored parameters of pesticides, herbicides, heavy metals and radioactivity.

2.2. Standards and reagents

Methanol was of analytical grade and supplied by Pronalab (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acids was purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). Phenolic standards (apigenin 6-*C*-glucoside, caffeic acid, chlorogenic acid, 3,4-dihydroxyphenylacetic acid, eriodictyol, hesperitin, isorhamnetin 3-*O*-rutinoside, kaempferol 3-*O*-glucoside, luteolin 7-*O*-glucoside, myricetin, protocatechuic acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, rosmarinic acid) were from Extrasynthese (Genay, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Preparation of the hydroalcoholic extract, infusion and decoction

Hydroalcoholic extractions were performed by stirring the plant material (1 g) with 30 mL of methanol/water (80:20, *v/v*) at 25 °C and 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 30 mL portion of the hydroalcoholic mixture. The combined extracts were evaporated at 35 °C

under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then further lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Infusions were prepared by adding 200 mL of boiling distilled water to the sample (1 g) and were left to stand at room temperature for 5 min, and then filtered under reduced pressure. Decoctions were performed by adding to 200 mL of distilled water to the sample (1 g), heated (heating plate, VELP scientific) and boiled for 5 min. The mixtures were left to stand for 5 min and then filtered under reduced pressure. The infusions and decoctions were frozen and lyophilized. The lyophilized hydroalcoholic extract, infusion and decoction were re-dissolved in methanol/water (80:20, v/v) and water, respectively, to obtain a stock solution of 20 mg/mL.

2.4. Evaluation of antioxidant activity

Four different *in vitro* assays were performed using solutions prepared by serial dilution of stock solution: scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, reducing power (measured by ferricyanide Prussian blue assay), inhibition of β -carotene bleaching and inhibition of lipid peroxidation in brain cell homogenates by TBARS (thiobarbituric acid reactive substances) assay.

DPPH radical-scavenging activity was evaluated by using a ELX800 microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{\text{DPPH}} - A_{\text{S}}) / A_{\text{DPPH}}] \times 100$, where A_{S} is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution (Chahdoura et al., 2014). Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+} , measuring the absorbance at 690 nm in the microplate Reader mentioned above. Inhibition of β -carotene bleaching was evaluated through the β -carotene/linoleate assay; the neutralization of linoleate free

radicals avoids β -carotene bleaching, which is measured by the formula: β -carotene absorbance after 2h of assay/initial absorbance) \times 100 (Chahdoura et al., 2014). Lipid peroxidation inhibition in pig (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) adduct was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively. The results were expressed in EC₅₀ values ($\mu\text{g/mL}$), i.e. sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power (Chahdoura et al., 2014).

2.5. Evaluation of antibacterial activity

To evaluate antibacterial activity, different bacteria were used (provided from American Type Culture Collection- ATCC), namely Gram positive species, such as, *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 35983), and Gram negative species, as *Escherichia coli* (ATCC 25922), *Klebsiella spp.*, *Pseudomonas aeruginosa* (ATCC 10145), *Enterococcus aerogenes* (ATCC 2048), *Proteus vulgaris* (ATCC 6380) and *Enterobacter sakazakii* (ATCC 29544). The antibacterial effect was evaluated using the disc diffusion halo test (NCCLS/CLSI & ANVISA, 2003). For that, each species was cultivated in a liquid medium, containing 30 mL of Tryptic Soy Broth (TSB), during 24h. After that, the concentration of each species was normalized for an optic density of 0.5 (corresponding approximately to 1×10^7 cells/mL) by absorbance determination at 600 nm. An aliquot of each species (300 μL) was spread in Tryptic Soy Agar (TSA) petri dishes. Then, an aliquot of 25 μL of each sample (decoction, infusion and hydroalcoholic extract dissolved in water- 20

mg/mL) was placed on sterile blank disc. The plates were incubated at 37 °C, during 24-48h. Antibacterial activity was measured using a qualitative method, based on disc diffusion assay. The qualitative results were converted in a semi-quantitative scale being classified the distinctness of the halo as: (-) absence of halo; (+) weak halo; (++) moderate halo; (+++) strong halo. Absence of halo concerning to 0.0 mm; weak halo between 3.0-7.0 mm; moderate halo 8.0-10.0 mm, and strong halo greater than 11.0 mm.

2.6. Analysis of phenolic compounds

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described ([Barros et al., 2013](#)). A Waters Spherisorb S3 ODS-2 C₁₈, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The injection volume was 100 μL. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out using diode array detector (DAD) using 280 at 370 nm as preferred wavelengths and a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve (1-100 μg/mL) for each available phenolic standard was constructed based on the UV signal: apigenin 6-C-glucoside ($y=223.22x+60.915$; $R^2=1$); caffeic acid ($y=611.9x-4.5733$; $R^2=0.999$); chlorogenic acid

($y=313.03x-58.2$; $R^2=0.999$); eriodictyol ($y=517.4x+268.26$; $R^2=0.992$); hesperitin ($y=178.59x + 154.29$; $R^2=0.999$); isorhamnetin 3-*O*-rutinoside ($y= 327.42x + 313.78$; $R^2=0.999$); kaempferol 3-*O*-glucoside ($y=288.55x-4.0503$; $R^2=1$); kaempferol 3-*O*-rutinoside ($y=239.16x-10.587$; $R^2=1$); luteolin 7-*O*-glucoside ($y=80.829x-21.291$; $R^2=0.999$); protocatechuic acid ($y=291.1x-6.4558$; $R^2=0.999$); quercetin-3-*O*-glucoside ($y=363.45x+117.86$; $R^2=0.999$), quercetin-3-*O*-rutinoside ($y=281.98x-0.3459$; $R^2=1$); rosmarinic acid ($y=336.03x+170.39$; $R^2=0.999$). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compounds from the same phenolic group. The results were expressed in mg per g of extract (the extraction yields to allow conversion for starting material are provided in **Table 1**).

2.7. Statistical analysis

All samples of thyme (infusion, decoction and hydroalcoholic extract) were prepared and analyzed in triplicate. The results, expressed as mean values and standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by Turkey's HSD Test with $\alpha = 0.05$, performed with SPSS v. 22.0 program.

3. Results and discussion

3.1. Evaluation of antioxidant activity

Table 1 shows the results obtained for antioxidant activity. In general, all the preparations revealed antioxidant potential, namely reducing power (RP), free radical scavenging activity (RSA), β -carotene bleaching inhibition (CBI) and lipid peroxidation inhibition (LPI) in brain cell homogenates. Decoction preparation showed the highest RSA and RP (EC_{50} values 112 and 101 $\mu\text{g/mL}$, respectively), while infusion revealed

the highest LPI (7 µg/mL). Concerning CBI, it was the hydroalcoholic extract that showed the highest value (32 µg/mL).

These results are in agreement with those of [Roby et al. \(2013\)](#) using methanol, ethanol, diethyl ether and hexane extracts of thyme from Egypt that related higher antioxidant capacity with higher contents in polyphenols, as obtained by using higher polarity solvents. Thus, in that study, the highest RSA was reported for the hydroalcoholic extract. The same correlation between higher contents in polyphenol compounds and antioxidant activity (measured by RSA, CBI and LPI assays) was also reported by [Kulisic et al. \(2006\)](#) who studied the infusions of cultivated and wild thyme from Croatia. Nevertheless, the results of RSA reported by those authors were not as good (with higher EC₅₀ values 300 µg/mL, for cultivated thyme and 450 µg/mL, for wild thyme) compared to the ones obtained in the present study (126.3 µg/mL).

3.2. Evaluation of antibacterial activity

Table 2 shows the results obtained in the screening of antibacterial activity of the infusion, decoction and hydroalcoholic extract prepared from thyme. Regarding Gram positive bacteria, although *S. aureus* and *S. epidermidis* belong to the same genus, thyme preparations only presented activity against *S. epidermidis*. The most pronounced effect was observed for Gram negative bacteria, with the order *E. coli* > *P. vulgaris*, *P. aeruginosa* > *E. aerogenes* = *E. sakazakii*. No effects were observed against *Klebsiella* spp. For Gram positive bacteria, decoction and infusion showed similar antibacterial activity, while hydroalcoholic extract revealed a lower activity. Regarding Gram negative bacteria, in general, decoction and hydroalcoholic extract had similar effect, except against *P. aeruginosa*, where infusion showed the lowest activity.

The majority of the studies available in the literature report antimicrobial activity of thyme essential oils, either individually (Oussalah, Caillet, Saucier, & Lacroix, 2007; Abdul, Hassan & Hassan, 2012) or combined with other essential oils (Al-Bayati 2008; Kon & Rai, 2012) or conventional antimicrobial agents. (Van Vuuren, Suliman, & Viljoen, 2009). A few experiments have reported the use of extracts. Carretto et al. (2007) described the interference of an aqueous extract of thyme on the adherence of *Streptococcus mutans* and *Candida albicans* to acrylic resin, and only verified a significant reduction of adherence in *C. albicans*, while there were no positive results for *S. mutans*. Moreover, Dababneh (2007) reported antimicrobial activity of thyme ethanolic extracts against six pathogens (*S. aureus*, *P. aeruginosa*, *E. coli*, *Enterobacter sp.* and *C. albicans*). Nevertheless, the extract concentrations were higher (100, 200, 400, 600, 800 and 1000 mg/mL) than the ones tested in the present work (20 mg/mL).

3.3. Identification and quantification of phenolic compounds

The phenolic profile of *T. vulgaris*, obtained after hydroalcoholic extraction, and recorded at 370 nm is shown in **Figure 1**; peak characteristics and tentative identities are presented in **Table 3**. Twenty-four compounds were detected, seven of which were phenolic acid derivatives and seventeen flavonoids. As for the phenolic acids, 3,4-dihydroxyphenylacetic acid (peak 1), protocatechuic acid (peak 2), 5-*O*-caffeoylquinic acid (peak 3), caffeic acid (peak 5) and rosmarinic acid (peak 18) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Peak 11 ($[M-H]^-$ at m/z 521) was identified as rosmarinic acid-glucoside, compound previously reported to occur in thyme (Nagy et al., 2011). Peak 22 ($[M-H]^-$ at m/z 537), consistent with the caffeic acid trimers salvianolic acids H/I and lithospermic acid A. Salvianolic acid I (i.e., 3'-*O*-(8''-*Z*-caffeoyl)rosmarinic

acid) was identified in *Thymus vulgaris* leaves by [Dapkevicius et al. \(2002\)](#) and further reported in dried thyme by [Nagy et al. \(2011\)](#). However, the fragmentation pattern of peak 22 matched better with the one described for lithospermic acid A than for salvianolic acid I by [Zeng et al. \(2006\)](#) and [Ruan et al. \(2012\)](#). Therefore, the compound was tentatively assigned to be lithospermic acid.

Flavonoids detected in the analysed sample belonged to the groups of flavones, flavonols and flavanones. Quercetin 3-*O*-rutinoside (peak 12), quercetin 3-*O*-glucoside (peak 13), luteolin 7-*O*-glucoside (peak 15) and eridictyol (peak 23) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards.

Quercetin 3-*O*-glucoside was previously reported in *Thymus vulgaris* ([Vergara-Salinas et al., 2012](#)), whereas luteolin 7-*O*-glucoside was identified in thyme infusions ([Kulisic et al., 2006](#)), leaves from *Thymus vulgaris* ([Hossain et al., 2011](#); [Vergara-Salinas et al., 2012](#)) and wild thyme (*T. serpyllum*) ([Miron et al., 2011](#)). The presence of eridictyol in different *Thymus* species was reported by [Kosar et al. \(2005\)](#), [Boros et al. \(2010\)](#) and [Miron et al. \(2011\)](#), and rutin (*i.e.*, quercetin 3-*O*-rutinoside) was also previously reported by [Boros et al. \(2010\)](#) and [Hossain et al. \(2010\)](#).

Peaks 6 and 9 ($[M-H]^-$ at m/z 449) were consistent with eridictyol *O*-hexoside, although the nature and position of the sugar residue could not be established. An eridictyol-glucoside was also found in thyme by [Nagy et al. \(2011\)](#). Peaks 7, 10 and 14 were identified as luteolin derivatives according to their UV and mass spectral characteristics. Peak 14 ($[M-H]^-$ at m/z 461) was assigned to luteolin 7-*O*-glucuronide, owing to the identification of that compound in leaves from *Thymus vulgaris* by [Dapkevicius et al. \(2003\)](#) and [Vergara-Salinas et al. \(2012\)](#), and wild thyme (*T. serpyllum*) by [Miron et al. \(2011\)](#). Peak 7 ($[M-H]^-$ at m/z 637) was tentatively identified as luteolin *O*-

diglucuronide and peak 10 ($[M-H]^-$ at m/z 447) was assigned to a luteolin hexoside. Peak 8 ($[M-H]^-$ at m/z 463) was assigned as quercetin *O*-hexoside and the presence of quercetin 7-*O*-glucoside in thyme was cited by [Roby et al. \(2013\)](#), which might correspond to peak 8 in our sample. Peak 17 showed the same mass spectral characteristics as peak 12 but a different UV spectrum with λ_{max} at 284 nm consistent with a flavanone, which allowed its assignment as a hesperetin *O*-rutinoside. [Vallverdú-Queralt et al. \(2014\)](#) revealed the presence of hesperetin 7-*O*-rutinoside, which could correspond to this peak. The presence of hesperetin as an aglycone in different *Thymus* species has also been cited by [Boros et al. \(2010\)](#).

Peak 16 ($[M-H]^-$ at m/z 477) presented characteristic UV and MS spectra consistent with an isorhamnetin hexoside. An undefined isorhamnetin 3-*O*-hexoside was also reported in thyme by [Hossain et al. \(2010\)](#). Peak 19 ($[M-H]^-$ at m/z 445) was tentatively assigned as apigenin *O*-glucuronide. Apigenin 7-*O*-glucuronide was reported in wild thyme by [Miron et al. \(2011\)](#), and an undefined apigenin-glucuronide was also found in dried thyme spices by [Nagy et al. \(2011\)](#). Similarly, peak 21 ($[M-H]^-$ at m/z 477, and MS^2 fragment ion at m/z 285) could be assigned as kaempferol *O*-glucuronide. Peak 20 ($[M-H]^-$ at m/z 607) yielded fragment ions at m/z 299 (-308 mu; loss of a rhamnosyl glucoside residue) and 284 (further loss of $-CH_3$), which allowed its assignment as methyl kaempferol *O*-rutinoside. As far as we know, methyl kaempferol derivatives have not been previously reported in thyme. Peak 24 ($[M-H]^-$ at m/z 283) could correspond to a methyl apigenin, as it was reported in thyme by [Hossain et al. \(2010\)](#). Finally, peak 4 ($[M-H]^-$ at m/z 593) was identified as apigenin-6,8-di-*C*-glucoside previously reported in thyme by [Vergara-Salinas et al. \(2012\)](#).

From the twenty-four phenolic compounds identified, rosmarinic acid (in all the preparations), luteolin 7-*O*-glucoside (in the hydroalcoholic extract) and luteolin 7-*O*-

glucuronide (in the infusion and decoction) were the most abundant phenolic acids and flavonoids found. Decoction presented the highest concentration of phenolic compounds (phenolic acids and flavonoids) followed by infusion and hydroalcoholic extract. This can also be related to the highest antioxidant (RSA and RP assays) and antibacterial activity obtained in decoction preparation. There are various publications reporting the phenolic composition of *T. vulgaris* from different origins and obtained by different extraction methodologies (Zheng & Wang 2001; Dapkevicius et al., 2002; Koşar et al., 2005; Proestos et al., 2005; Kulisic, Dragovi-Uzelac, & Milos, 2006; Boros et al., 2010; Hossain et al., 2010; Miron et al., 2011; Nagy et al., 2011; Vergara-Salinas et al., 2012; Berdowska et al., 2013; Roby et al., 2013), although the phenolic profiles reported are different in each case, presenting only similarities in some of the compounds identified in our study.

Zheng and Wang (2001) and Berdowska et al. (2013) studied a sample of *T. vulgaris* from the USA and Poland, after extraction with acetone and water (boiling water and further hydrolysis), respectively. Both identified caffeic and rosmarinic acids; these compounds were also identified under this study and rosmarinic acid was also the most abundant phenolic compound detected. They also detected some flavonoids, such as hispidulin, luteolin, luteolin 7-*O*-glucuronide; the last one was also identified in the present study. Vergara-Salinas et al. (2012) presented the phenolic composition of *T. vulgaris* from Chile obtained after pressurized hot water extraction. These authors identified seventeen compounds (five phenolic acids and twelve flavonoids), seven of which were also detected in the studied samples. Proestos et al. (2005) analysed a sample of *T. vulgaris* from Greece, using aqueous methanol extract with a further hydrolysis of the extract. These authors identified and quantified five phenolic acids (gentisic, caffeic, *p*-coumaric, syringic and *p*-hydroxybenzoic acids) and three

flavonoids (apigenin, luteolin and epicatechin). These authors did not identify rosmarinic acid which is the major phenolic acid found in the studied *T. vulgaris*. Furthermore, [Koşar et al. \(2005\)](#) identified four flavonoids (luteolin glucuronide, eriodictyol, luteolin and apigenin) and one phenolic acid (rosmarinic acid), in a methanolic extract of *T. vulgaris* from Finland. Rosmarinic acid was the most abundant phenolic compound found. [Roby et al. \(2013\)](#) and [Kulisic et al. \(2006\)](#) studied *T. vulgaris* samples from Egypt (methanolic extract) and Croatia (infusion preparation), respectively. In the methanolic extract of *T. vulgaris*, eight phenolic acids were identified, being cinnamic acid, a phenolic acid related compound, the major molecule found. These authors also identified five flavonoids, in which the aglycone apigenin was the most abundant. [Kulisic et al. \(2006\)](#) showed results for the infusion of *T. vulgaris*, identifying three phenolic acids and six flavonoids, and in accordance to the results presented herein, rosmarinic acid was the most abundant compound found. Coincidences with the phenolic compounds detected by other authors (i.e., [Dapkevicius et al., 2002](#); [Boros et al., 2010](#); [Hossain et al., 2010](#); [Miron et al., 2011](#); [Nagy et al., 2011](#)) are discussed above in this paper.

The variations observed in the phenolic profiles of samples from different origins could be explained by growing conditions (soil, climate, rainfall, altitude), harvesting, processing, among others, which directly interfere with the content of chemical constituents (e.g., phenolic compounds) and, consequently, in their therapeutic effects ([Naghdi Badi, Yazdani, Ali, & Nazari, 2004](#)).

In summary, the use of infusion and decoction of thyme (rich in phenolic compounds, either phenolic acids or flavonoids), as complementary of daily food, could provide considerable benefits for health, not only in the treatment of diseases related to reactive

species production and oxidative stress, but also against bacterial infections. These benefits conferred by thyme can be achieved by both internal and external use and, at the recommended doses, it is safe and no adverse reactions have been described. Nevertheless, further experiments are required in order to explore the mechanisms of action involved.

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Table 1. Extraction yield (%) and antioxidant activity (EC₅₀ values, µg/mL) of infusion, decoction and hydroalcoholic extract of *Thymus vulgaris* L. (mean ± SD).

	Infusion	Decoction	Hydroalcoholic extract
Extraction yield	12.11 ± 1.62	10.96 ± 2.18	6.98 ± 0.31
DPPH scavenging activity (RSA)	126.3±3.9 ^b	112.3±5.4 ^c	279.2±1.8 ^a
Reducing power (RP)	116.8±2.2 ^b	100.7±2.4 ^c	183.0±3.9 ^a
β-carotene bleaching inhibition (CBI)	86.4±7.9 ^b	207.9±6.1 ^a	31.6±0.8 ^c
TBARS inhibition (LPI)	7.1±0.3 ^c	14.1±0.6 ^a	12.9±0.2 ^b

EC₅₀ values correspond to the sample concentration providing 50% of antioxidant activity; RP was expressed as concentration giving absorbance of 0.500. In each row different letters mean significant differences (p<0.05).

Table 2. Antibacterial activity of infusion, decoction and hydroalcoholic extract of *Thymus vulgaris* L. (mean \pm SD)

	Infusion	Decoction	Hydroalcoholic extract
Gram positive bacteria			
<i>Staphylococcus aureus</i>	-	-	-
<i>Staphylococcus epidermidis</i>	++	++	+
Gram negative bacteria			
<i>Escherichia coli</i>	++	+++	+++
<i>Klebsiella</i> spp.	-	-	-
<i>Pseudomonas aeruginosa</i>	+	++	+
<i>Enterobacter aerogenes</i>	+	+	+
<i>Enterobacter sakazakii</i>	+	+	+
<i>Proteus vulgaris</i>	+	++	++

(-) absence of halo; (+) weak halo; (++) moderate halo; (+++) stronger halo.

Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{\max}), mass spectral data, identification and quantification of phenolic compounds in and hydroalcoholic extracts, infusion and decoction of *Thymus vulgaris* L. (mean \pm SD)

Peak	Rt (min)	λ_{\max} (nm)	Molecular ion [M-H] ⁻ (<i>m/z</i>)	MS ² (<i>m/z</i>)	Identification	Quantification (mg/g extract)		
						Infusion	Decoction	Hydroalcoholic
1	4.85	280	197	179(38),151(9),135(100)	3,4-dihydroxyphenylacetic acid	1.72 \pm 0.09	1.90 \pm 0.16	1.57 \pm 0.02
2	6.22	258,sh292	153	123(2),109(100)	Protocatechuic acid	0.73 \pm 0.01	0.85 \pm 0.04	0.76 \pm 0.02
3	8.38	330	353	191(100),179(5),173(4),161(4),135(2)	5- <i>O</i> -Caffeoylquinic acid	1.46 \pm 0.06	1.07 \pm 0.01	0.87 \pm 0.02
4	11.02	336	593	473(24),383(9), 297(24),253(3)	Apigenin 6,8-di- <i>C</i> -glucoside	1.57 \pm 0.02	1.43 \pm 0.15	nd
5	11.61	328	179	135(100),107(4)	Caffeic acid	1.82 \pm 0.05	2.20 \pm 0.09	1.84 \pm 0.06
6	14.02	282	449	287(100)	Eriodictyol <i>O</i> -hexoside	0.69 \pm 0.02	0.70 \pm 0.07	tr
7	15.51	350	637	461(55),285(38)	Luteolin <i>O</i> -diglucuronide	9.96 \pm 0.08	10.11 \pm 0.70	1.66 \pm 0.13
8	17.79	354	463	301(100)	Quercetin <i>O</i> -hexoside	1.18 \pm 0.10	1.46 \pm 0.25	1.48 \pm 0.13
9	17.83	284	449	287(100)	Eriodictyol <i>O</i> -hexoside	1.39 \pm 0.04	1.79 \pm 0.32	1.85 \pm 0.12
10	18.77	346	447	285(100)	Luteolin <i>O</i> -hexoside	4.27 \pm 0.13	4.67 \pm 0.32	2.19 \pm 0.09
11	19.17	320	521	359(100),197(21),179(35),161(74),135(13)	Rosmarinic acid glucoside	3.45 \pm 0.06	3.16 \pm 0.31	3.24 \pm 0.02
12	20.00	352	609	301(100)	Quercetin 3- <i>O</i> -rutinoside	1.31 \pm 0.10	1.46 \pm 0.13	0.75 \pm 0.04
13	20.55	350	463	301(100)	Quercetin 3- <i>O</i> -glucoside	1.01 \pm 0.06	1.12 \pm 0.04	0.82 \pm 0.01
14	20.97	348	461	285(100)	Luteolin 7- <i>O</i> -glucuronide	26.59 \pm 0.06	25.63 \pm 0.18	8.79 \pm 0.22
15	21.03	346	447	285(100)	Luteolin 7- <i>O</i> -glucoside	14.95 \pm 0.30	16.62 \pm 0.58	26.43 \pm 0.30
16	22.37	352	477	315(100)	Isorhamnetin <i>O</i> -hexoside	0.56 \pm 0.06	0.74 \pm 0.06	0.32 \pm 0.00
17	23.19	284	609	301(100)	Hesperetin 7- <i>O</i> -rutinoside	4.40 \pm 0.02	7.43 \pm 0.06	4.92 \pm 0.19
18	24.33	330	359	197(99),179(90),161(100),135(53)	Rosmarinic acid	23.52 \pm 0.09	23.98 \pm 0.09	22.26 \pm 0.53

9	25.46	340	445	269(100)	Apigenin <i>O</i> -glucuronide	3.04 ± 0.29	3.12 ± 0.09	1.64 ± 0.13	
10	26.17	336	607	299(100),284(35)	Methyl kaempferol <i>O</i> -rutinoside	1.58 ± 0.05	1.66 ± 0.11	1.46 ± 0.07	
11	27.25	344	461	285(100)	Kaempferol <i>O</i> -glucuronide	1.91 ± 0.15	2.10 ± 0.07	1.15 ± 0.01	
12	28.46	328	537	493(100),359(89),313(17),295(64),197(27),179(54),161(82),135(82)	Lithospermic acid A	nd	2.64 ± 0.61	4.38 ± 0.11	
13	31.02	288	287	151(100),135(95)	Eriodictyol	0.11 ± 0.01	0.11 ± 0.02	0.66 ± 0.15	
14	32.48	336	283	268(100)	Methyl apigenin	0.36 ± 0.01	0.65 ± 0.03	0.50 ± 0.02	
						Total phenolic acids	32.69 ± 0.14 ^c	35.81 ± 1.13 ^a	34.92 ± 0.69 ^b
						Total flavonoids	74.87 ± 1.22 ^b	80.79 ± 2.81 ^a	54.60 ± 1.00 ^c
						Total phenolic compounds	107.56 ± 1.08 ^b	116.60 ± 3.95 ^a	88.59 ± 1.69 ^c

In the last three rows different letters mean significant differences ($p < 0.05$).

Figure 1. Phenolic profile of *Thymus vulgaris* hydroalcoholic extract recorded at 370 nm.