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Simultaneous characterization and quantification of phenolic compounds in *Thymus x citriodorus* using a validated HPLC–UV and ESI–MS combined method



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ABSTRACT

Thymus x citriodorus is a Lamiaceae plant extensively cultivated in the Mediterranean region and used for centuries in culinary and in traditional medicine. The present work describes the detailed phenolic composition of *T. x citriodorus* for the first time, by means of HPLC–DAD, ESI–MS and MSⁿ and nuclear magnetic resonance (NMR) analyses. The ethanolic extract of *T. x citriodorus* was analyzed by reversed phase HPLC. The method of analysis was also validated concerning its linearity, instrumental and method precision (for repeatability, immediate precision and intermediate precision) and accuracy (absolute recovery study). The technique was combined with electrospray mass spectrometry in order to identify the phenolic compounds and the structure of the main phenolics was also confirmed by NMR analysis. The in-house validated HPLC–DAD method showed good linearity for the tested reference compounds as well as satisfactory repeatability and immediate precision values, for both instrument and method. Furthermore, the satisfactory results of intermediate precision analysis and recovery assays indicated that the chromatographic method could be used to quantify the main phenolic compounds of *T. x citriodorus* with adequate precision and accuracy. The extract was rich in rosmarinic acid (10.4 ± 0.6 mg/g extract) that is a widespread phenolic acid in *Thymus* plants, but also in luteolin-7-*O*- α -glucuronide (12 ± 2 mg/g extract), that was herein reported in *Thymus* for the first time. Other novel compounds comprised one eriodictyol dihexoside with *O*-glycosidic linkages, two eriodictyol-*O*-monohexosides, one quercetagenin dimethyl ether-*O*-hexoside, one naringenin-*O*-hexoside and chrysoeriol-7- β -*O*-glucoside. Having in mind the health-promoting properties reported in literature for some of the main polyphenols found in *T. x citriodorus*, we suggest that this plant has a high potential for being used as a functional food.

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1. Introduction

In recent years, several industries have shown a great interest in edible plants and in their bioactive compounds because of their potential applications, including as functional food and nutraceuticals (Wijngaard, Hossain, Rai, & Brunton, 2012). *Thymus* L. is a large genus belonging to the Lamiaceae family, which comprises 300–400 endemic species widely distributed in the entire World, in particular in the Mediterranean region. These plants are perennial, herbaceous, tender and of simple small leaves with ramified and prostrated branches, forming a shrub with uncountable branches, typically reaching a height of 10 to 30 cm (Reddy, Angers, Gosselin, & Arul, 1998).

Many *Thymus* species are known as culinary herbs and have been cultivated for usage in the confection of several dishes and in flavoring salads, soups, stews and sauces. Additionally, *Thymus* species are used in infusion form as medicinal plants because of their biological and pharmacological properties, which include expectorant, anti-asthmatic, bronchiolytic, anti-septic, antispasmodic, analgesic, antimicrobial, and antioxidant (Gião et al., 2007; Mata et al., 2007; Pinto et al., 2006). It is believed that some of these beneficial activities are due to their volatile constituents and thus, their essential oil composition has been the focus of many investigations (Horvath, Szabo, Hethelyi, & Lemberkovics, 2006; Omidbaigi, Sefidkon, & Hejazi, 2005). In contrast, there are only a limited number of studies focusing the composition of other bioactive phytochemicals of *Thymus* plants, such as their phenolic compounds. According to the few studies on this topic, the hydrophilic extracts of dried thyme plants contain caffeic acid and its oligomers [rosmarinic acid, 3'-*O*-(8"-caffeoyl) rosmarinic acid, lithospermic acid and methyl rosmarinic], flavones

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(apigenin, luteolin, luteolin-7-*O*- β -glucuronide, luteolin-7-*O*-glucoside, 6-hydroxyluteolin glycosides, chrysoeriol and polymethoxyflavones), flavanones (naringenin, naringenin-7-*O*-glucoside, narirutin, eriodictyol, eriodictyol-7-*O*-glucoside, isosakuranetin, eriocitrin and hesperidin), and the flavanol taxifolin (Dorman, Bachmayer, Kosar, & Hiltunen, 2004; Fecka & Turek, 2008).

Thymus x citriodorus, or lemon thyme, is one of the most used *Thymus* in culinary (The Herb Society of America, 2003). The plant is used as an ingredient for confection of several recipes of starter (Cheese-stuffed Nasturtiums), snacks, sauces (Chili Oil, Soyer's Recipe for Goose Stuffing) and different meat (Meat Stuffing for Duck), fish (Fish Aspic Jelly) or vegetarian (Spinach Frittata with Herbs) dishes. Additionally, it is used in jellies and desserts (Lemon Thyme Jelly) for confection of soups (Cream of Porcini Soup, Thick Giblet Soup) and consumed in fresh salads as well as in marinades for grilled fish, chicken and roast duck, potatoes and carrots (Celtnet, 2013). Besides its culinary usage, *T. x citriodorus* is also vastly consumed in the form of tea, for medicinal proposals.

Despite the widespread culinary consumption of *T. x citriodorus* and its claimed health benefits, the detailed knowledge of its phenolic constituents remains unknown. The present work used a combination of HPLC with ESI-MS/MSⁿ and nuclear magnetic resonance (NMR) analysis, in order to contribute for the knowledge of the phenolic constituents in *T. x citriodorus*.

2. Experimental

2.1. Plant material

T. x citriodorus plants were purchased from ERVITAL (Castro de Aire, Portugal) as a mixture of leaves and stems. The plants have been cultivated under an organic regime and the collected aerial parts were dried at 25–30 °C in a ventilated incubator for approximately 5 days.

2.2. Solvents and reagents

n-Hexane was purchased from Pronalab (Lisbon, Portugal), the analytical grade reagents formic acid and ethanol were obtained from Panreac (Barcelona, Spain), methanol and acetonitrile with HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Eriodictyol-7-*O*-glucoside, luteolin-7-*O*-glucoside, naringenin-7-*O*-glucoside, apigenin-7-*O*-glucoside, chrysoeriol and rosmarinic acid were obtained from Extrasynthese (Genay Cedex, France).

2.3. Extraction of phenolic compounds

Aerial parts (leaves and stems) of *T. citriodorus* (5 g) were grounded and defatted three times with 150 mL *n*-hexane, for 30 min each at room temperature. The residue was extracted with 150 mL of an 80% ethanol solution (v/v) at room temperature, for 30 min. The resulting mixture was filtered and the residue was re-extracted in the same conditions for four times. At the end, the filtrated solutions were combined, concentrated, frozen at –20 °C and freeze-dried. The dried extract was stored in vacuum desiccator at dark for subsequent use. Each sample was prepared in triplicate.

2.4. HPLC–DAD analyses

2.4.1. HPLC apparatus and chromatographic conditions

The HPLC analysis was performed on a Varian 9010 separation module equipped with PDA Varian Prostar detector, under the general conditions previously described, with some modifications (Pereira, Silva, Domingues, & Cardoso, 2012). Data acquisition and remote control of the HPLC system were done by Varian Star chromatography Workstation® (Lake Forest, CA, USA) software. The column was a

250 mm × 4 mm id, 5 μ m bead diameter, end-capped Nucleosil C18 (Macherey-Nagel) and its temperature was maintained at 30 °C.

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% (v/v) of formic acid in water and solvent B consisted of acetonitrile, which were degassed and filtrated, using a 0.2 μ m nylon filter (Whatman, USA) before use. The solvent gradient consisted in a series of linear gradients: from 10 to 30% of solvent B over 20 min, from 30 to 100% of solvent B over 5 min, decreasing to 10% of solvent B after 5 min followed by the return to the initial conditions. The flow rate was 1 mL/min and the injected volume was equal to 10 μ L.

2.4.2. Method in-house validation

The HPLC method used to detect and quantify the phenolic compounds was validated for linearity, precision (assays performed for repeatability and intermediate precision) and accuracy (absolute recovery study).

2.4.2.1. Linearity, limits of detection and of quantification. An external standard calibration methodology was applied. Five solutions with different concentrations of eriodictyol-7-*O*-glucoside (10.0–135.9 μ g/mL), naringenin-7-*O*-glucoside (5.0–67.9 μ g/mL), luteolin-7-*O*-glucoside (45.3–300.0 μ g/mL), apigenin-7-*O*-glucoside (2.5–160.0 μ g/mL) and rosmarinic acid (14.9–120.0 μ g/mL) were prepared by consecutive dilutions from a stock solution. The analyses were performed in triplicate and the results were plotted for evaluating the linear relationship between the peak areas of each phenolic standard. ANOVA was used to assess the statistical significance of each linear regression model being the quality of the fitted models evaluated by their R² values. The statistical significances of the slope and of the intercept values were evaluated by a t-test. Finally, the regression data were subjected to a likelihood ratio test of equality (covariance analysis) to infer about inter-day variability of the calibration curves in order to avoid establishing a new calibration curve whenever a quantification procedure was needed. Statistic analyses were performed using the SPSS 17 Standard Version software (SPSS INC.) at a 5% significance level. Detection (LOD) and quantification (LOQ) limits were calculated using the parameters of the calibration curves, being defined as 3.3 and 10 times the value of the regression error divided by the slope, respectively (Ermer & Miller, 2005; Snyder, Kirkland, & Dolan, 2010).

2.4.2.2. Precision (repeatability and intermediate precision). Both instrumental and method precisions were evaluated to verify the repeatability of the system and of the proposed method (extraction procedure followed by chromatographic analysis).

The instrumental system precision was studied using three standard solutions, containing eriodictyol-7-*O*-glucoside (10, 30 or 80 μ g/mL), naringenin-7-*O*-glucoside (5, 40 or 50 μ g/mL), luteolin-7-*O*-glucoside (45, 100 or 300 μ g/mL), apigenin-7-*O*-glucoside (10, 80 or 160 μ g/mL), and rosmarinic acid (15, 30 or 150 μ g/mL), which corresponded to low, middle and high concentration levels. Each solution was injected, under the working conditions, 5 times on the same day to evaluate the repeatability of the instrumental system (i.e., intra-day variation, considering only within day variations). Further, the immediate precision of the system was evaluated by determining the variability of the responses of the injections of the three standard solutions, injected 3 times per day in three consecutive days (i.e., inter-day variation, considering within and between day variations). The instrumental precision was assessed by calculating the relative standard deviation percentage (%RSD).

The method precision was inferred based on the evaluation of repeatability and immediate precision. For that, an ethanolic extract from *T. x citriodorus* was obtained according to the work conditions, from a sample of *T. x citriodorus* (5.0010 ± 0.0001 g) resulting in 0.75 g of lyophilized extract. Part of the lyophilized extract (50.6 mg)

was re-dissolved into 5 mL of methanol and then injected 5 times in the same day and 3 times per day in three consecutive days for method repeatability and immediate precision assessment, respectively.

The intermediate precision of the method was studied using two extracts obtained from the same plant according to the procedure previously described (extracted, stirred, filtered, re-extracted, combined, concentrated under reduced pressure, frozen and finally freeze-dried separately). Each extract was injected in triplicate in three consecutive days. Subsequently, the intermediate precision of the method was evaluated by calculating the %RSD value of each phenolic compound detected, considering within and between day variations as well as between extraction variations.

2.4.2.3. Accuracy. The accuracy of the proposed method was studied by evaluating the absolute recovery, which studies the retrieval of standards added to a biological sample, that was subjected to all steps of the extraction procedure (extraction, filtration, re-extraction, concentration and freeze-dried). Two levels of luteolin-7-*O*-glucoside and rosmarinic acid (the available phenolic standards corresponding to natural phenolic constituents of the *T. x citriodoros*) were added to 2.04 ± 0.02 g of *T. x citriodoros* dry plant samples. The quantity of each substance recovered in relation to the added amount was calculated, taking into account the yield of the extraction procedure.

2.4.3. Identification and quantification of the phenolic compounds

The identification of the phenolic compounds of the ethanolic extracts of *T. x citriodoros* was based on the UV-vis spectrum of the HPLC fractions together with their analysis by electrospray ionization mass spectrometry (ESI-MS and ESI-MSⁿ). This latter was performed on a Linear Ion trap LXQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA), following the procedure previously described (Pereira, Silva, et al., 2012).

When standards were available, the identification of the compounds was further confirmed by comparison of their HPLC-DAD retention time, UV-vis profile and ESI-MSⁿ data to those of the phenolic standards. Moreover, the structure of some compounds (luteolin-5- β -*O*-glucoside, luteolin-7- α -*O*-glucuronide, chrysoeriol-7- β -*O*-glucoside, apigenin-7- β -*O*-glucuronide and rosmarinic acid) was further confirmed by NMR analysis. The HPLC freeze-dried collected fractions (2–3 mg) were dissolved in DMSO-*d*₆ and the ¹H spectra were recorded at 298 K on a Bruker Avance 500 spectrometer operating at 500.13 MHz. 2D NMR (heteronuclear single quantum coherence, using gradient pulses for selection i.e. (¹H,¹³C) gHSQC, heteronuclear multiple quantum coherence,

using gradient pulses for selection i.e. gHMBC) spectra were acquired in the same experimental conditions as previously described (Pereira, Domingues, Silva, & Cardoso, 2012). ¹³C NMR chemical shift assignments were made from the projections of the heteronuclear HSQC and HMBC experiments.

The quantification of phenolic compounds was performed by peak integration using the HPLC-DAD external standard method, with the most close reference compound available to that of the major compound in each HPLC eluting peak. More concretely, fraction 1 (eriodictyol-di-*O*-hexoside) and fraction 3 (eriodictyol-*O*-hexoside) were quantified using eriodictyol-7-*O*-glucoside as the reference compound. The same reference compound was also used to quantify fraction 2 (eriodictyol-*O*-hexoside and quercetagenin dimethyl ether *O*-hexoside) as, in accordance to the UV-vis and MS spectra, the eriodictyol-*O*-hexoside was the major phenolic compound of that fraction. In a similar approach, fractions 5 (naringenin-*O*-hexoside and eriodictyol-*O*-hexuronide) and 6 (luteolin-7- α -*O*-glucuronide plus luteolin-7-*O*-glucoside) were quantified using naringenin-7-*O*-glucoside and luteolin-7-*O*-glucoside as reference, respectively. Fraction 4 (luteolin-5- β -*O*-glucoside) and fraction 8 (apigenin-7- β -*O*-glucuronide) were respectively quantified using luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside as reference, while rosmarinic acid was used as the reference for quantification of phenolic compounds in fractions 9 (rosmarinic acid) and 10 [(3'-*O*-(8''-*Z*-caffeoyl)rosmarinic acid)]. Moreover, and according to the maximum UV absorption, the quantification of compounds eluting in fractions 1, 2, 3 and 5 was performed by HPLC-DAD at 280 nm while the compounds eluting in fractions 4, 6, 8, 9 and 10 were quantified at 340 nm.

3. Results and discussion

3.1. Identification of the phenolic compounds of the ethanolic extract of *T. x citriodoros*

Overall, the identified phenolic compounds of the ethanolic extract from *T. x citriodoros* enclosed rosmarinic acid and another less common phenolic acids, as well as derivatives of common flavonoids, including the flavones luteolin, chrysoeriol and apigenin, the flavanones eriodictyol and naringenin, and the flavonol quercetagenin (Table 1). A description of the *T. x citriodoros* phenolic composition and the comparison to that previously described for other *Thymus* plants, will be discussed below in detail.

Table 1
Identification of HPLC-eluting fractions by HPLC/DAD and ESI/MS from *T. x citriodoros*.

Fraction number	RT (min)	λ_{\max}	[M – H] [–]	Main ESI-MS ⁿ (abundance)	Compound
1	4.3	283, 327	611	MS ² [611]: 449(100), 287(15); MS ³ [449]: 287(100), 151(<1); MS ⁴ [287]: 269(2), 151(100); MS ⁵ [151]: 107	Eriodictyol-di- <i>O</i> -hexoside
2	6.8	283, 327	387	MS ² [387]: 369(15), 225(5), 207(100), 163(10), 119(1); MS ³ [207]: 163; MS ⁴ [163]: 109	5'-Hydroxyjasmonic acid 5'- <i>O</i> -hexoside
			449	MS ² [449]: 287; MS ³ [287]: 151; MS ⁴ [151]: 107	Eriodictyol- <i>O</i> -hexoside
3	7.3	283, 327	507	MS ² [507]: 489(20), 471(10), 345(35), 327(100), 315(5); MS ³ [327]: 312(100), 167(20); MS ³ [345]: 327(100), 315(15), 309(20), 287(5)	Quercetagenin dimethyl ether <i>O</i> -hexoside
			449	MS ² [449]: 287(100), 269(<1), 151(1); MS ³ [287]: 269(4), 161(<1), 151(100), 125(4), 107(1); MS ⁴ [151]: 107	Eriodictyol- <i>O</i> -hexoside
4	8.6	248, 342	447	MS ² [447]: 285(100); MS ³ [285]: 243(60), 241(100), 199(100), 175(50), 151(10)	Luteolin-5- β - <i>O</i> -glucoside
5	9.1	283, 340	433	MS ² [433]: 271(100); MS ³ [271]: 227(1), 177(10), 151(100), 107(2)	Naringenin- <i>O</i> -hexoside
			463	MS ² [463]: 301(20), 287(100); MS ³ [287]: 151(100), 135(<1), 125(<1)	Eriodictyol- <i>O</i> -hexuronide
6	9.6	254, 267	461	MS ² [461]: 285(100); MS ³ [285]: 241(95), 217(60), 199(60), 175(60), 151(20)	Luteolin-7- α - <i>O</i> -glucuronide
			447	MS ² [447]: 285; MS ³ [285]: 243(50), 241(100), 199(60), 175(50), 151(15)	Luteolin-7- <i>O</i> -glucoside
7	10.9	245, 338	461	MS ² [461]: 446(1), 341(4), 323(3), 299(100); MS ³ [299]: 284(100); MS ⁴ [284]: 256(40), 151(5); MS ⁵ [256]: 239(4), 227(100), 211(20), 200(10), 122(60), 94(2)	Chrysoeriol-7- β - <i>O</i> -glucoside
			445	MS ² [445]: 269(100), 175(5); MS ³ [269]: 225(5), 183(1)	Apigenin-7- β - <i>O</i> -glucuronide
9	11.5	290, 328	359	MS ² [359]: 223(15), 197(25), 179(30), 161(100), 133(4); MS ³ [179]: 161(25), 151(<1), 135(100)	Rosmarinic acid
			537	MS ² [537]: 493; MS ³ [493]: 359(100), 357(15), 313(10), 295(3), 269(<1), 247(<1), 179(1), 161(1); MS ⁴ [359]: 249(5), 223(10), 197(15), 179(25), 161(100), 135(5)	3'- <i>O</i> -(8''- <i>Z</i> -Caffeoyl)rosmarinic acid

3.1.1. Phenolic acid derivatives

In accordance with literature data, the rosmarinic acid, which was herein identified by its retention time, UV–vis spectrum, ESI–MSⁿ (Table 1) and NMR (Table 2) data, represented a major HPLC fraction in the *T. x citriodorus* ethanolic extract (fraction 9, Fig. 1). To our knowledge, this compound has been previously detected in *Thymus serpyllum*, *Thymus sipyleus*, *Thymus quinquecostatus* and *Thymus vulgaris* L., and has been shown to account between 3.4 and 22 mg/g of dry plant, in the latter species (Pereira & Cardoso, 2013). Besides this phenolic acid, the *T. x citriodorus* ethanolic extract also contained an uncommon caffeoyl derivative of rosmarinic acid (fraction 10), which was assigned based on its UV–vis spectrum, as compared to that of rosmarinic acid, plus interpretation of its ESI–MSⁿ fragmentation pattern (Dapkevicius et al., 2002). Most probably, this compound corresponds to 3'-O-(8''-Z-caffeoyl)rosmarinic acid, which has been previously detected in the leaves of *T. vulgaris* L. (Dapkevicius et al., 2002).

3.1.2. Flavones

Flavones were also detected as major phenolic component of the *T. x citriodorus* ethanolic extract. In more detail, the extract contained three luteolin derivatives, which were eluted in fractions 4 and 6 ([M – H][–] ion at *m/z* 447 in fraction 4 and [M – H][–] ions at *m/z* 461 and *m/z* 447 in fraction 6). The [M – H][–] ion at *m/z* 447 in the latter fraction was assigned to luteolin-7-*O*-glucoside, since the retention time and MSⁿ data matched with those of the reference compound. In turn, the [M – H][–] ion at *m/z* 447 in fraction 4 was assigned to luteolin-5-β-*O*-glucoside, based on the gathered 1D (Table 2) and 2D NMR spectral data. From these spectra it was possible to assign the major part of the ¹H and ¹³C resonances, mainly obtained from the connectivities found in the HMBC spectrum of this compound (the connectivity between H-1'' and C-5 allowed us to assign the sugar position on the flavone skeleton). The coupling constant of the H-1 of the sugar moiety (*J* = 7.3 Hz) indicates the presence of the β-anomer. To our

knowledge, for *Thymus* plants the latter luteolin derivative has only been previously detected in *T. sipyleus* and in *T. praecox* (Ozgen et al., 2011).

Moreover, the HPLC–DAD–ESI–MSⁿ analysis allowed to assign the [M – H][–] ion at *m/z* 461 in fraction 6 to luteolin-*O*-hexuronyl, as the product ion at *m/z* 285 was obtained by the loss of 176 Da (Fig. 2A). Additional information on the sugar moiety (glucuronic acid), as well as its linkage position on the flavone skeleton (7–) was elucidated by 1D and 2D NMR analysis (see Fig. 2B for ¹H RMN and gHSQC spectra). These NMR spectra allowed us to assign the major part of the proton and carbon resonances (Table 2), but due to the small quantity of the sample we could not find the HMBC correlation between H-1'' from the sugar residue and the C-7 of the flavone. However, the assigned chemical shifts are compatible with a 7-glucuronide (Agrawal & Bansal, 1989; Lu & Foo, 1999). H-1'' of the sugar residue appears as a broad singlet, which is only compatible with an α-configuration. Note that despite the fact that luteolin-*O*-glucuronide has been previously described in several *Thymus* plants (Justesen, 2000; Miron, Plaza, Bahrim, Ibanez, & Herrero, 2011), only the 7-*O*-β-isomer has been previously identified by NMR analysis (Fecka & Turek, 2008; Ozgen et al., 2011).

Besides the luteolin derivatives, the *T. x citriodorus* ethanolic extract also contained another flavone hexuronyl derivative (fraction 8, [M – H][–] ion at *m/z* 445). In this particular case, the MS² spectrum showed a main ion at *m/z* 269, and the latter followed a fragmentation pattern consistent to that of apigenin. According to the NMR determinations (Guvenalp, Ozbek, Kuruuzum-Uz, Kazaz, & Demirezer, 2009) the [M – H][–] ion at *m/z* 445 was assigned to apigenin-7-β-*O*-glucuronide, which, for the *Thymus* genus, has only been previously reported in *T. vulgaris* L. and *T. serpyllum* (Justesen, 2000; Miron et al., 2011).

Notably our study also allowed to detect, for the first time in *Thymus* plants, a hexoside derivative of the methylated flavone chrysoeriol. This compound was eluted in fraction 7 and appeared

Table 2
Chemical shifts (δ) of phenolic compounds of *T. x citriodorus* ethanolic extract, which eluted in fractions 4, 6, 7, 8 and 9 (in DMSO-d₆).

Compound	4		6		7		8		9		
	Luteolin-5-β- <i>O</i> -glucoside		Luteolin-7-α- <i>O</i> -glucuronide		Chrysoeriol-7-β- <i>O</i> -glucoside		Apigenin-7-β- <i>O</i> -glucuronide		Rosmarinic Acid		
Atom	¹³ C (ppm)	¹ H (ppm)	¹³ C (ppm)	¹ H (ppm)	¹³ C (ppm)	¹ H (ppm)	¹³ C (ppm)	¹ H (ppm)	Atom	¹³ C (ppm)	¹ H (ppm)
2	161.2	–	164.5	–	160.8	–	164.3	–	1	130.4	–
3	105.3	6.50 (s)	103.1	6.76 (s)	106.2	6.58 (s)	103.1	6.88(s)	2	114.5	6.65 (br s)
4	176.6	–	182.0	–	176.1	–	ni	–	3	144.7	ni
5	158.7	–	161.2	13.00 (s)	ni	12.97 (s)	ni	ni	4	143.5	ni
6	105.2	6.73 (br s)	99.5	6.45 (br s)	99.5	6.43 (br s)	104.4	6.81 (J 2.0 Hz)	5	116.4	6.59 (d, J 7.6 Hz)
7	ni	OH 8.45 (s)	162.8	–	ni	ni	ni	ni	6	120.0	6.47 (br d, J 7.6 Hz)
8	98.5	6.61 (br s)	94.6	6.80 (br s)	94.6	6.83 (br s)	98.1	6.73 (J 2.0 Hz)	7	36.3	3.00 (d, J 12.4 Hz) 2.70 (d, J 12.4 and 10.1 Hz)
9	ni	–	157.1	–	ni	–	ni	–	8	76.2	4.79 (br d, J 10.1 Hz)
10	107.4	–	105.4	–	ni	–	ni	–	9	ni	ni
1'	121.1	–	121.4	–	123.2	–	ni	–	1'	125.7	–
2'	112.9	7.34 (s)	113.6	7.43 (br d)	112.8	7.40 (br s)	128.4	7.97 (d, J 8.7 Hz)	2'	114.5	7.02 (br s)
3'	145.8	OH 5.12 (br s)	145.8	9.48 (br s)	146.8	–	116.0	6.94 (d, J 8.7 Hz)	3'	148.2	ni
4'	149.8	OH 5.12 (br s)	150.0	10.01 (br s)	150.7	ni	161.5	–	4'	145.7	ni
5'	115.8	6.85 (d, J 7.8 Hz)	115.9	6.90 (d, J 8.4 Hz)	110.5	7.07 (d, J 8.2 Hz)	ni	ni	5'	115.4	6.74 (d, J 8.4 Hz)
6'	118.6	7.35 (d, J 7.8 Hz)	119.2	7.45 (d, J 8.4 Hz)	118.4	7.49 (br d, J 8.2 Hz)	ni	ni	6'	121.0	6.94 (d, J 8.4 Hz)
Sugar									7'	143.5	7.34 (d, J 15.9 Hz)
1''	105.1	4.67 (d, J 7.3 Hz)	99.5	5.19 (br s)	103.1	4.68 (d, J 7.0 Hz)	99.5	5.03 (d, J 7.3 Hz)	8'	115.1	6.16 (d, J 15.9 Hz)
2''	73.6	*	73.0	*	ni	ni	ni	ni	9'	166.2	–
3''	75.6	*	75.2	*	ni	ni	ni	ni			
4''	69.8	*	72.9	*	ni	ni	ni	ni			
5''	77.6	*	72.8	*	ni	–	ni	–			
6''	60.8	*	ni	–	ni	ni	ni	ni			

* Under the peak of water.

ni – not identified.

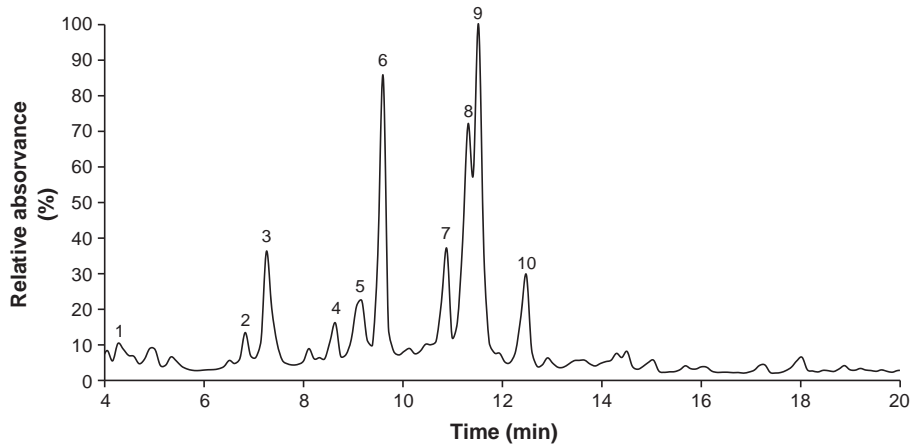


Fig. 1. Chromatographic profile of the ethanolic extract obtained from *T. x citriodorus* at 280 nm. The numbers in the figure correspond to the fractions collected for further analysis by ESI-MSⁿ and NMR.

in the ESI-MS spectrum as the $[M - H]^-$ ion at m/z 461 that fragmented to a main product ion at m/z 299 (– 162 Da, loss of hexose). Moreover, the fragmentation pattern of this product ion was similar to that of chrysoeriol (Plazonic et al., 2009). This information

was corroborated by the NMR analysis, which also allowed us to assign the exact structure of this flavone to chrysoeriol-7- β -O-glucoside (Table 2). The β -configuration of the sugar residue is based on the coupling of the H-1', $J = 7.3$ Hz.

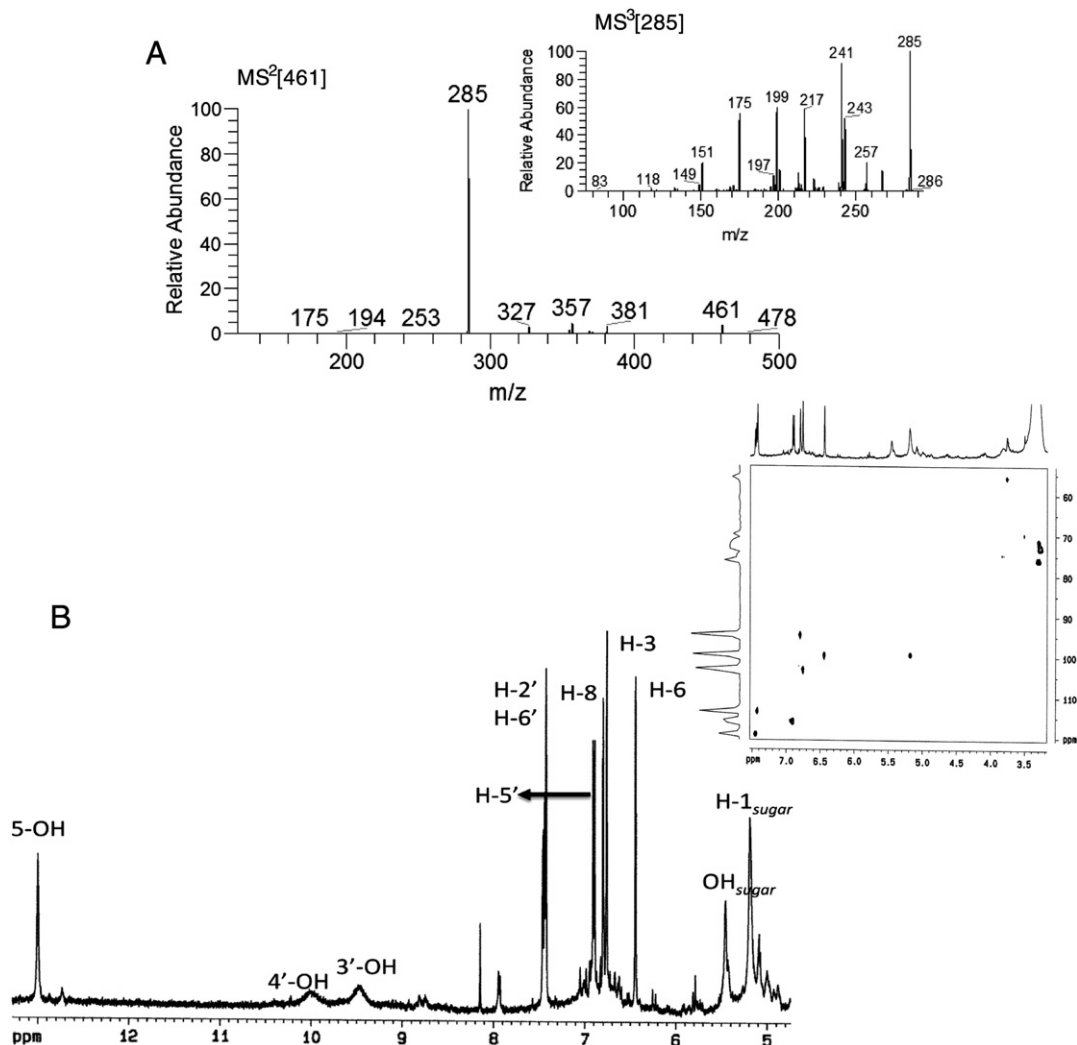


Fig. 2. Mass spectrum of (A) ESI-MS² of the $[M - H]^-$ ion at m/z 461 and (inset) ESI-MS³ of the $[M - H]^-$ ion at m/z 285; (B) expansion of the ¹H NMR and (inset) gHSQC spectra of fraction 6 eluted on HPLC-DAD.

3.1.3. Flavanones

Three glycoside derivatives of eriodictyol have been previously described in the genus *Thymus*, namely the eriodictyol-7-*O*-glucoside (Fecka & Turek, 2008), the eriodictyol-7-*O*-rutinoside (Wang, Li, Ho, Peng, & Ho, 1998) and the eriodictyol-7-*O*-glucuronide (Justesen, 2000). Nevertheless, the results now obtained show that three of the four eriodictyol derivatives detected in the ethanolic extract of *T. x citriodorus* differ from those previously reported for the other species of *Thymus*. Indeed, the ESI-MS analysis of fraction 1 showed that the $[M - H]^-$ ion at m/z 611 and its MS^2 and MS^3 fragmentation data revealed the loss of two hexose molecules (product ions at m/z 449 and m/z 287). Moreover, the ESI- MS^3 spectrum of the $[M - H]^-$ ion at m/z 449 was similar to that of authentic eriodictyol-7-*O*-glucoside and hence, these data indicates the elution in fraction 1 of an eriodictyol dihexoside with *O*-glycosyl linkages. Note that, flavonoid diglycosides with *O*-linkages (di-*O*-glycosides and *O*-diglycosides), can be distinguished by their product ion spectra (Vukics & Guttman, 2008), through the analysis of the $[M - H - 162]^-$, $[M - H - 180]^-$ and $[M - H - 324]^-$ ions. A high relative intense product ion $[M - H - 162]^-$ and the absence of $[M - H - 180]^-$ at the MS^2 spectrum, as observed for the ion at m/z 611, indicate the presence of a flavonoid di-*O*-hexoside and thus, the present results confirm the detection, for the first time in *Thymus*, of an eriodictyol-di-*O*-hexoside.

Eriodictyol-*O*-monohexosides were detected in fractions 2 and 3. As observed in Table 1, the ESI-MS spectra of both fractions showed a $[M - H]^-$ ion at m/z 449, and their MS^2 spectrum revealed the ion at m/z 287, which corresponds to the eriodictyol aglycone. The UV-vis data of these two peaks were similar to that of the eriodictyol-7-*O*-glucoside standard, but fractions 2 and 3 eluted before that compound, indicating that two new eriodictyol-*O*-hexosides must be present in *T. x citriodorus*. In fact, to our knowledge, eriodictyol-7-*O*-glucoside is the only described glucoside derivative of eriodictyol in Lamiaceae family. Besides the above described *O*-hexosyl derivatives of eriodictyol, the *T. x citriodorus* ethanolic extract also contained an *O*-hexuronol of this flavanone, but this appeared only as a minor constituent of fraction 5. Eriodictyol-*O*-glucuronide has been previously found in thyme and wild thyme (Justesen, 2000; Miron et al., 2011), but no quantitative information has been delivered on that compound on those plants. Besides eriodictyol derivatives, the flavanone naringenin-*O*-hexoside was also identified as a phenolic constituent of the *T. x citriodorus* ethanolic extract. Its $[M - H]^-$ ion was observed in fraction 5 at m/z 433 and this fragmented to the ion at m/z 271 (-162 Da). Moreover, the MS^3 spectrum of the ion at m/z 271 (main ions at m/z 227, 151 and 107), indicated a correspondence to authentic naringenin (Fabre, Rustan, de Hoffmann, & Quetin-Leclercq, 2001). Despite the fact that the detailed structure of this compound could not be accomplished in the present study, for sure, this does not correspond to the unique naringenin glucoside described so far in *Thymus*, i.e. the naringenin-7-*O*-glucoside (Fecka & Turek, 2008), as it eluted before the naringenin-7-*O*-glucoside standard (10.5 min).

3.1.4. Flavonols

In accordance to the previous studies focusing on *Thymus* plants, the ethanolic extract of *T. x citriodorus* was very poor in flavonols. In the present study, the unique flavonol was detected as a minor component of fraction 2. Its corresponding molecular ion was observed for m/z 507 and its MS^2 spectrum showed product ions formed by the loss of a hexose moiety [ion at m/z 345 (-162 Da) and ion at m/z 327 (simultaneous loss of 162 and 18 Da)]. Considering that the ion at m/z 345 can diagnose the quercetagenin dimethyl ether (Parejo, Jauregui, Viladomat, Bastida, & Codina, 2004), the overall data suggests the presence of the flavonol quercetagenin dimethyl ether-*O*-hexoside in *T. x citriodorus*. Methyl derivatives of quercetagenin, namely quercetagenin 3,7-dimethyl ether, have been described to occur in the Lamiaceae family (Grayer et al., 2010), however this is the first time that this class of compounds has been found in the genus *Thymus*.

3.2. HPLC method – validation and quantification of phenolic compounds by HPLC-DAD

For the five used phenolic standards, the adjusted R^2 values were around 0.999, suggesting a good linearity of the analytical method in the concentration range tested (Table 3) and showing that the HPLC method allows the quantification of the evaluated phenolic compounds, in case of their presence in the plant samples. All the calibration linear models were significant ($P < 0.001$) as well as their slope values ($P < 0.001$) and the intercept values ($P < 0.003$, except for apigenin-*O*-glucoside with $P = 0.228$). Finally, the covariance analysis for each phenolic standard (data not shown) indicated that the calibration curves of one week interval were not statistically different ($P \geq 0.330$), meaning that the same calibration curve could be used during at least one week for quantification purposes. Table 3 also shows the LOD and LOQ values, which were always lower than the lowest standard concentration tested in the dynamic interval of the calibration curve, indicating a satisfactory sensitivity towards each phenolic standard.

The relative standard deviation percentage (%RSD) values of repeatability and of immediate precision assays were carried out with the five phenolic standards, regarding the instrumental precision evaluation. In repeatability, %RSD values varied from 2 to 5% and from 0.3 to 2%, for the retention time and the peak area, respectively. Concerning the immediate precision, the %RSD values of the retention times and peak areas were between 3–4% and 1–2%, respectively. These results are similar to those described by Du et al. (2010) and Gobbo-Neto and Lopes (2008) for phenolic compounds in Lamiaceae and Asteraceae plants, respectively. Since the %RSD values were lower than 5%, it is possible to conclude that the chromatographic instrument presents a satisfactory precision (Ermer & Miller, 2005).

The %RSD values for repeatability, immediate precision and intermediate precision assays of the method (extraction plus HPLC analysis) are given in Table 3. As the values for the first two parameters were lower than 12%, it can be concluded that the precision of the method was satisfactory. Regarding intermediate precision, the %RSD values varied from 6% to 41%, being in general lower than 20%. The highest values [33% and 41% for eriodictyol-7-*O*-glucoside and 3'-*O*-(8"-Z-Caffeoyl) rosmarinic acid, respectively] could be due to the low content of these compounds (Table 3), as well as to the nature of the extraction process and the complexity of the chromatographic analysis (Aldai, Osoro, Barrón, & Nájera, 2006). In fact, depending on the samples complexity, %RSD values up to 20% are acceptable for quantitative chromatographic analysis of residual analytes (Ribani, Bottoli, Collins, Jardim, & Melo, 2004). Therefore, it can be stated that the proposed method is able to quantify the major phenolic compounds detected in *T. x citriodorus* samples with overall satisfactory precision.

Regarding the two absolute recovery assays carried out (addition of 0.986 mg of luteolin-7-*O*-glucoside and 0.121 mg of rosmarinic acid or 1.548 mg of luteolin-7-*O*-glucoside and 0.555 mg of rosmarinic acid to 2.04 ± 0.02 g of dry plant samples, respectively) recoveries were of 120% and 60% or 121% and 79% for luteolin-7-*O*-glucoside and rosmarinic acid, respectively, with %RSD values between 2% and 4%. These results are acceptable as, depending on the complexity of the chromatographic method and the sample matrix, recovery values between 50% and 120% are adequate if their %RSD values are lower than 15% (Ribani et al., 2004).

The mean total phenolic contents (mg/g of extract) of the *T. x citriodorus* plant were evaluated from the intermediate precision assays and are presented in Table 3. According to the results, the identified phenolic compounds accounted for 44 mg/g of the ethanolic extract (or 7.5 mg/g of dried plant). Similarly to other *Thymus* species, such as *T. vulgaris* L. and *T. spicata* (Dorman et al., 2004; Fecka & Turek, 2008), the ethanolic extract of *T. x citriodorus* was mostly enriched in rosmarinic acid (10.4 ± 0.6 mg/g extract). Still, our results also showed

Table 3
Linearity, LOD, LOQ and instrumental and method precisions.

Instrumental precision study							
Standard compound	Range concentration (µg/mL)	n ^a	Slope ^b (area counts/mg)	Intercept ^b (area counts/mg)	R ²	LOD (µg/mL)	LOQ (µg/mL)
E-70-G	10.0–135.9	5	144(±1). 10 ⁶	−43(±9). 10 ⁴	0.9999	2.0	6.2
N-70-G	5.0–67.9	5	1797(±6). 10 ⁵	−19(±2). 10 ⁴	0.9999	1.0	3.0
L-70-G	45.3–300.0	5	663(±7). 10 ⁵	−4(±1). 10 ⁵	0.9984	12.4	37.7
A-70-G	2.5–160.0	5	848(±8). 10 ⁵	−1(±6). 10 ⁴	0.9988	7.3	22.1
RA	14.9–120.0	5	1343(±9). 10 ⁵	−27(±6). 10 ⁴	0.9995	3.3	9.9
Instrumental precision study (intra- and inter-days variability of standard solutions injections)							
Standard compound	Concentration (µg/mL)	%RSD					
		Repeatability (n = 5)		Immediate precision (n = 9)			
		Retention time (min)	Peak area	Retention time (min)	Peak area		
E-70-G	10	1.1	1.4	4.8	2.1		
	30	1.3	0.9	3.1	2.1		
	80	2.5	1.5	3.6	1.7		
N-70-G	5	2.3	2.1	4.3	2.6		
	40	3.3	0.7	3.1	1.8		
	50	1.3	0.5	3.1	1		
L-70-G	45	0.6	1.0	4.4	0.9		
	100	1.3	0.5	3.4	1.2		
	300	3.5	0.2	3.7	1.6		
A-70-G	10	0.2	1.6	3.4	2.5		
	80	3.4	1.1	4.3	1.6		
	160	0.9	1.1	3.9	2		
RA	15	2.5	0.7	3.5	3.2		
	30	2.1	0.7	3	0.8		
	150	1.2	0.9	3.1	1.3		
Method precision study (intra- and inter-days variability of extractions obtained from a <i>Thymus</i> sample)							
Identified compound	Standard compound	Mean content ^c	%RSD for compound concentrations				
Fraction number	Compound name	(used to quantify)	(mg/g extract)	Repeatability	Immediate precision	Intermediate precision	
				(n = 5)	(n = 9)	(n = 18)	
1	Eriodictyol-di-O-hexoside	E-70-G	0.71 ± 0.07	5	7	11	
2	Eriodictyol-O-hexoside	E-70-G	1.3 ± 0.4	10	12	33	
3	Eriodictyol-O-hexoside	E-70-G	3.7 ± 0.5	4	7	12	
4	Luteolin-5-β-O-glucoside	L-70-G	3.2 ± 0.5	2	4	16	
5	Naringenin-O-hexoside	N-70-G	1.8 ± 0.2	6	6	9	
6	Luteolin-7-α-O-glucuronide	L-70-G	12 ± 2	4	5	20	
8	Apigenin-7-β-O-glucuronide	A-70-G	9 ± 2	6	7	20	
9	Rosmarinic acid	RA	10.4 ± 0.6	6	5	6	
10	3'-O-(8"-Z-Caffeoyl)rosmarinic acid	RA	2.3 ± 0.9	4	4	41	
Total	–	–	44 ± 4	–	–	–	

E-70-G, eriodictyol-7-O-glucoside; N-70-G, naringenin-7-O-glucoside; L-70-G, luteolin-7-O-glucoside; A-70-G, apigenin-7-O-glucoside; RA, rosmarinic acid.

^a Number of points used for the regression of standard solutions. Injections were done in triplicate.

^b The standard deviation in the slope and intercept of the regression line is shown in parentheses.

^c Mean values ± standard deviations.

that in contrast to the majority of the previously studied *Thymus* plants, the non-common isomeric form of luteolin-O-hexoside, i.e. the luteolin-7-α-O-glucuronide, as well as apigenin-7-β-O-glucuronide, were also major phenolic constituents of the extract, accounting for 12 ± 2 mg/g extract and 9 ± 2 mg/g extract, respectively.

4. Conclusions

The phenolic composition of an ethanolic extract of *T. x citriodorus* was described in detail for the first time, by means of HPLC–DAD plus ESI–MS, MSⁿ and NMR analysis. The in-house validated HPLC–DAD method showed good linearity for the tested reference compounds as well as satisfactory repeatability and immediate precision values, for both instrument and method. Furthermore, the satisfactory results of intermediate precision analysis and recovery assays indicated that the chromatographic method could be used to quantify the main phenolic compounds of *T. x citriodorus* with adequate precision and accuracy. The fractionation of the ethanolic extract by HPLC–DAD reversed phase and the analysis of the collected freeze-dried fractions by electrospray mass spectrometry in the negative mode allowed us to

identify thirteen phenolic compounds, which include the phenolic acid rosmarinic acid and one of its derivatives, as well as derivatives of the flavones luteolin, chrysoeriol and apigenin, of the flavanones eriodictyol and naringenin and of the flavonol quercetagenin. Similarly to other *Thymus* species, rosmarinic acid represented a major phenolic constituent of the *T. x citriodorus* ethanolic extract. However, our results also suggest that this plant species produces high amounts of non-common *Thymus* phenolics, including the 7-α-O-glucuronide derivative of luteolin and apigenin-7-β-O-glucuronide. As the majority of these compounds are described in literature as health-benefit compounds, we propose that this *Thymus* species can be used as nutraceutical agent with potential interest for food and pharmaceutical industries.

Abbreviations used

CID	collision-induced dissociation
DAD	diode array
ESI–MS	electrospray ionization–mass spectrometry
MS ⁿ	tandem mass spectrometry
GAE	gallic acid equivalent

HPLC	high-performance liquid chromatography
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
RSD	relative standard deviation

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