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Effects of gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Mentha* x *piperita* L.

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ABSTRACT

The aim of the present study was to evaluate the effects of gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Menta* × *piperita* L. (methanolic extracts), used in traditional medicine. Thirteen and fourteen phenolic compounds, including caffeoyl derivatives and flavonoid glycosides, were detected in *T. vulgaris* and *Mentha piperita*, respectively, none of which was affected by the irradiation dose used (10 kGy). Furthermore, the irradiation up to 10 kGy did not change the cytotoxic properties of peppermint samples on tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2), whereas thyme samples irradiated at 10 kGy increased their cytotoxicity in the assayed tumor cell lines compared with samples submitted to 2 and 5 kGy. All in all, the dose of 10 kGy was considered as suitable to be applied for the purpose of disinfestation and microbial decontamination of these plants without modifying their phenolic composition and bioactive properties.

1. Introduction

Phytochemicals present in medicinal plants have revealed to be beneficial for the prevention of various diseases due to its capacity to act in multiple biological mechanisms (Zhang et al., 2011). Its antioxidant potential acts in the neutralization of reactive oxygen species (ROS) that cause damaging changes in the cells (Bajpai, Agrawal, Bang, & Park, 2015; Jain, Jain, Jain, Jain, & Balekar, 2010). The increasing interest in the use of phytochemicals is due to their natural origin, the possibility to be ingested through the diet, easy acquisition and also due to their reduced adverse effects (Dillard & German, 2000). The use of medicinal plants for the treatment of different diseases dates back to ancient times, revealing satisfactory results as anti-inflammatory, antimicrobial, antimutagenic, anticancer and antioxidant agents.

These effects are in part explained by the presence of phytochemicals such as phenolic compounds (Wojdyło, Oszmiański, & Czemerys, 2007).

Phenolic compounds are secondary metabolites ubiquitously distributed in plants (Jabri-Karoui, Bettaieb, Msaada, Hammami, & Marzouk, 2012; Wojdyło et al., 2007). They include a large group of biologically active compounds, with over 8000 individual molecules described, having at least one aromatic ring with one or more hydroxyl groups attached, being able to vary from small molecules to large and complex ones. These compounds usually appear in their natural sources as esters and glycosides (Roby, Sarhan, Selim, & Khalel, 2013; Vallverdú-Queralt et al., 2014).

Many species of plants recognized for their medicinal properties and beneficial impact on health contain these metabolites, such as e.g., *Thymus vulgaris* L. (thyme) and *Mentha* × *piperita* L. (peppermint) (Zgorka & Glowniak, 2001; Kapp et al., 2013), belonging to the Lamiaceae family. Thyme is a plant widely used in folk medicine and its essential oil has shown bioactive properties because of its composition in bioactive metabolites (mixture of monoterpenes, being the main compounds thymol), such as antimicrobial, anti-

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inflammatory, expectorant, spasmolytic, antioxidant and hepatoprotective activities (Fecka & Turek, 2008; Fachini-Queiroz et al., 2012; Martins et al., 2015; Nikolić et al., 2014; Gavarić et al., 2015). Similarly, peppermint is a perennial herb that is also commonly used in traditional medicine, mainly consumed as teas. It has been associated to antioxidant, antitumor, antimicrobial, hypoallergenic and immunomodulatory effects, as well as benefits for the digestive tract (Grigoleit & Grigoleit, 2005; McKay & Blumberg, 2006; Singh, Shushni, & Belkheir, 2015). Its essential oil is also well known and widely used in food, pharmaceutical and cosmetic industries, because of presenting biological activity against several organisms (Moghaddam, Pourbaige, Tabar, Farhadi, & Hosseini, 2013; Sharma & Sharma, 2013).

The commercialization and use of medicinal plants must accomplish certain rules regarding to decontamination (Haleem, Salem, Fatahallah, & Abdelfattah, 2015). Irradiation is increasingly recognized as a suitable approach to decontaminate natural matrices and preserve their components (Kume, Furuta, Todoriki, Uenoyama, & Kobayashi, 2009). In particular, gamma irradiation was approved for disinfection and microbial control in various food products and supplements by the Food and Drug Administration (FDA), being a procedure technically and economically viable and physically safe with a powerful antimicrobial effect (Mizani, Sheikh, Ebrahimi, Gerami, & Tavakoli, 2009). This decontamination method has interesting advantages that present it as a good alternative to other methods, namely for aromatic herbs (Pereira et al., 2015a). The majority of the studies with gamma irradiated herbs are related with the effects on nutritional composition and antioxidant properties. In this work we aimed at evaluating the effects on cytotoxicity and phenolic compounds using two Lamiaceae herbs (thyme and peppermint) as case-studies.

2. Materials and methods

2.1. Herbs and samples irradiation

Samples of *T. vulgaris* L. (thyme) and *Mentha* × *piperita* L. (peppermint) were provided as dry leaves by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal). After confirmation of the taxonomical identification, the samples were divided into four groups: control (non-irradiated, 0 kGy), and samples irradiated with different doses (2, 5 and 10 kGy).

A Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with total activity 140 TBq (3.77 kCi), was used for sample irradiations, in May 2015. During the irradiation procedure, the dose and dose rate were estimated using a chemical solution sensitive to ionizing radiation, with an Amber Perspex dosimeters (Batch X, from Harwell Company, Didcot, Oxfordshire, UK), and a Fricke dosimeter, respectively (ASTM, 1992; Pereira et al., 2015a).

The estimated doses for thyme samples were 2.4 ± 0.1 kGy, 5.5 ± 0.2 kGy and 10.4 ± 0.5 kGy; and 2.2 ± 0.3 kGy, 5.7 ± 0.21 kGy and 10.3 ± 0.4 kGy for peppermint samples. The dose rates and dose uniformity ratios (D_{max}/D_{min}) were, 1.2 kGy/h and 1.1 respectively. For simplicity, the values 0, 2, 5 and 10 kGy were considered for the doses of non-irradiated and irradiated groups, respectively.

2.2. Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic compound standards (apigenin-6-*C*-glucoside, caffeic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside and rosmarinic acid) were from Extrasynthese (Genay, France). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all with PA purity, were purchased from Panreac S.A. (Barcelona, Spain) (proanalysis), in order to prepare the acid aqueous Fricke dosimeter solution.

2.3. Preparation of the extracts

The dried samples of *T. vulgaris* and *Mentha* × *piperita* were reduced to a fine dried powder (20 mesh) and mixed to obtain homogeneity. To prepare the methanolic extracts, each sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and then filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of 25 mL of methanol (25 °C at 150 rpm) for another 1 h. Subsequently, the combined extracts were evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) until dryness.

2.4. Evaluation of cytotoxic properties

2.4.1. General

For cytotoxicity evaluation the extracts were redissolved in water, with a final solution of 8 mg/mL and diluted to different concentrations, and ellipticine was used as positive control. The results were calculated as GI_{50} values (sample concentration that inhibited 50% of the net cell growth).

2.4.2. In tumor cell lines

The human tumor cell lines used were: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines were plated in a 96-well plate, at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 and 1.0×10^4 cells/well for HeLa and HepG2) and were allowed to attach for 24 h. Afterwards, various extract concentrations were added to the cells and incubated during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100 μL) was used in order to bind the adherent cells and further incubated for 60 min at 4 °C. After the incubation period, the plates were washed with deionised water and dried and sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 μL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Guimarães et al., 2013).

2.4.3. In non-tumor cells

A freshly harvested porcine liver, obtained from a local slaughter house, was used in order to obtain the cell culture, designated as PLP2. The liver tissues were rinsed in Hank's balanced salt solution containing penicillin (100 U/mL), streptomycin (100 μg/mL) and divided into 1 × 1 mm^3 explants. A few of these explants were transferred to a tissue flasks (25 cm^2) containing DMEM medium supplemented with fetal bovine serum (FBS, 10%), nonessential amino acids (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL) and incubated at 37 °C with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cell cultivation was continuously monitored using a phase contrast

microscope. When confluence was reached, the cells were subcultured and plated in 96-well plate (density of 1.0×10^4 cells/well) containing DMEM medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). The growth inhibition was evaluated using the SRB assay, previously described (Guimarães et al., 2013).

2.5. Analysis of phenolic compounds

The extracts obtained above were analysed using a HPLC chromatograph (Hewlett–Packard 1100, Agilent Technologies, Santa Clara, CA, US) with a double online detection using a diode array detector (DAD) with 280, 330 and 370 nm as preference wavelengths, and a mass spectrometer (MS) equipped with an ESI source and a triple quadrupole-ion trap mass analyser, which was connected to the HPLC system via the DAD cell outlet (Barros et al., 2013). The DAD was coupled to an HP Chem Station (rev. A.05.04) and the MS was controlled by the Analyst 5.1 software. The separation was achieved using a Spherisorb S3 ODS-2 C_{18} column (3 μm, 4.6 × 150 mm) thermostatted at 35 °C, using a gradient elution with the following solvents: 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient established was 15% B 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% B for 10 min, and re-equilibration of the column (10 min), using a flow rate of 0.5 mL/min. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS), employed to show full scan spectra, and enhanced product ion (EPI) analysis. Air (zero graded) was used as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen functioned as the curtain (20 psi) and collision gas (medium). The ion spray voltage was set at −4500 V and spectras were recorded in negative ion mode between *m/z* 100 and 1700. The settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V. Compounds were tentatively identified comparing the obtained information (retention times, UV–vis and mass spectra) with available data reported in the literature and by comparison with standard compounds, when available. For the quantification a calibration curve for each available phenolic standard (apigenin-6-*C*-glucoside, caffeic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside, rosmarinic acid) was constructed based on the UV signal (maximum absorption of each standard compound lambda max). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of another compound from the same phenolic group. The results were expressed as mg per g of extract.

2.6. Statistical analysis

For each one of the species two samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. In the case of phenolic composition, a Student's *t*-test was used to determine the significant difference among two different samples, with $\alpha = 0.05$. This analysis was carried out using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Cytotoxic properties of extracts from non-irradiated and irradiated samples

The results obtained in the evaluation of the cytotoxic properties of thyme and peppermint (extracts prepared from non-irradiated and irradiated samples) against four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) and against non-tumor cells are given in Table 1. All samples showed cytotoxicity, and these results are in agreement with a previous study performed by Berdowska et al. (2013) that used dried aqueous extracts of *T. vulgaris* from Poland, reporting its cytotoxicity in MCF-7 (breast carcinoma) tumor cell line. Lv et al. (2012) also evaluated the antiproliferative activity of a peppermint extract against the human tumor cell line HT-29, describing promising results.

The thyme sample irradiated at 10 kGy showed cytotoxicity for MCF-7, HeLa and HepG2 cell lines similar to the control sample, and higher (lower GI_{50} values) than the toxicity revealed by the samples irradiated at 2 and 5 kGy. Regarding peppermint, no significant differences were observed when different doses were applied, with the exception of MCF-7 cell line, where a higher cytotoxicity was observed in the control sample (0 kGy). In relation to the toxicity for non-tumor cells, none of the samples presented hepatotoxicity ($GI_{50} > 400$ μg/mL).

3.2. Comparative analysis of the phenolic compounds in non-irradiated and irradiated samples

As, in general, no significant differences were found in the cytotoxic properties of the extracts prepared from samples irradiated at three different doses (2, 5 and 10 kGy). Nonetheless, control samples and samples irradiated at the highest dose (10 kGy) were used for phenolic compounds analysis, due to the higher efficiency of higher doses in the decontamination process.

Thirteen and fourteen phenolic compounds were identified in the methanolic extracts of thyme and peppermint, respectively. Tables 2 and 3 present data related to the phenolic compounds identification (retention time, λ_{max} in the visible region, molecular ion, main fragment ions in MS^2, tentative identification and quantification) obtained by HPLC-DAD-ESI/MS analysis for both species. Fig. 1 shows the phenolic compounds profile in thyme and peppermint, recorded at 280 nm.

Compounds 2 (apigenin 6,8-di-*C*-glucoside), 3 (caffeic acid), 6 (rosmarinic acid hexoside), 7 (luteolin-7-*O*-glucuronide), 8 (luteolin-7-*O*-glucoside), 10 (rosmarinic acid), 12 (lithospermic acid A) and 13 (eriodictyol) in thyme were already described and tentatively identified in a previous study carried out by our research group, but using a different commercial sample (Martins et al., 2015). Moreover, the majority of the mentioned compounds have been previously cited in thyme samples (Boros et al., 2010; Costa et al., 2012; Dapkevicius et al., 2002; Fecka & Turek, 2008; Hossain, Rai, Brunton, Martin-Diana, & Barry-ryan, 2010; Roby et al., 2013; Vallverdú-Queralt et al., 2014; Vergara-Salinas, Perez-Jiménez, Torres, Agosin & Pérez-Correa, 2012). Compounds 1, 4, 5, 9 and 11 were not described in our previous study (Martins et al., 2015), which may be due to the existence of different composition and/or distribution of phenolic compounds depending on the part tissue, the origin of the plant and the edaphoclimatic conditions (Boros et al., 2010; Costa et al., 2012). Based on their UV and mass spectra, compounds were tentatively identified as caffeic acid hexoside (peak 1), methyleriodictyol *O*-pentosylhexoside (peak 4) and quercetin-*O*-glucuronide (peak 5). Peak 9 ($[M-H]^-$ at *m/z* 537) showed similar UV and mass spectra characteristics as lithospermic acid A (peak 12), although it was eluted at a different retention

Table 1
Cytotoxicity of thyme and peppermint extracts prepared from non-irradiated and irradiated samples.

	Doses			
	0 kGy	2 kGy	5 kGy	10 kGy
Thyme				
MCF-7 (breast carcinoma)	88 ± 7^{b}	104 ± 6^{a}	106 ± 10^{a}	83 ± 9^{b}
NCI-H460 (non-small cell lung cancer)	294 ± 12^{a}	276 ± 16^{ab}	297 ± 11^{a}	262 ± 4^{b}
HeLa (cervical carcinoma)	161 ± 6^{b}	189 ± 13^{a}	191 ± 9^{a}	160 ± 13^{b}
HepG2 (hepatocellular carcinoma)	103 ± 10^{a}	110 ± 13^{a}	106 ± 8^{a}	100 ± 10^{a}
Hepatotoxicity PLP2 (non-tumor cells)	>400	>400	>400	>400
Peppermint				
MCF-7 (breast carcinoma)	114 ± 12^{b}	175 ± 15^{a}	150 ± 4^{ab}	154 ± 7^{ab}
NCI-H460 (non-small cell lung cancer)	226 ± 11^{a}	224 ± 2^{a}	213 ± 20^{a}	229 ± 16^{a}
HeLa (cervical carcinoma)	221 ± 13^{a}	206 ± 11^{a}	211 ± 21^{a}	214 ± 12^{a}
HepG2 (hepatocellular carcinoma)	98 ± 9^{a}	115 ± 9^{a}	106 ± 11^{a}	111 ± 12^{a}
Hepatotoxicity PLP2 (non-tumor cells)	>400	>400	>400	>400

Positive control (Ellipticine) - MCF-7: 1.21 ± 0.02; NCI-H460: 1.03 ± 0.09; HeLa: 0.91 ± 0.11; HepG2: 1.10 ± 0.09; PLP2: 2.29 ± 0.18. GI_{50} values (μg/mL) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

Table 2
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in thyme extracts prepared from non-irradiated and irradiated samples (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Type of identification	Quantification (mg/g)		*t*-Students test *p*-value
							0 kGy	10 kGy	
1	7.3	320	341	179(100),135(88)	Caffeic acid hexoside	References 1,2,3,4/DAD/MS	1.7 ± 0.1	1.5 ± 0.1	0.988
2	10.8	338	593	473(20),383(33),353(27),297(5)	Apigenin 6,8-di-*C*-glucoside	Reference 5/ DAD/MS	3.45 ± 0.04	3.31 ± 0.04	0.212
3	11.3	326	179	135(100)	Caffeic acid	Reference 5/ Standard/DAD/ MS	2.69 ± 0.01	2.5 ± 0.4	0.258
4	17.4	284,336sh	595	301(47),286(100)	Methyleriodictyol-*O*-pentosylhexoside	DAD/MS	3.12 ± 0.01	1.9 ± 0.4	0.005
5	18.1	350	461	301(100)	Quercetin-*O*-glucuronide	DAD/MS	0.4 ± 0.1	0.35 ± 0.03	0.742
6	18.8	322	521	359(100),197(13),179(36),161(62),135(21)	Rosmarinic acid hexoside	Reference 5/ DAD/MS	16.8 ± 0.1	14.7 ± 0.2	0.378
7	20.0	348	461	285(100)	Luteolin-7-*O*-glucuronide	Reference 5/ DAD/MS	8.4 ± 0.2	7.1 ± 0.2	0.381
8	20.8	350	447	285(100)	Luteolin-7-*O*-glucoside	Reference 5/ Standard/DAD/ MS	3.34 ± 0.01	3.2 ± 0.1	0.003
9	23.4	286,320sh	537	493(20),359(70),295(5),197(13),179(28),161(100), 135(63)	Caffeic acid trimer	DAD/MS	9.1 ± 0.1	8.0 ± 0.1	0.027
10	24.0	330	359	197(17),179(35),161(100),135(29)	Rosmarinic acid	Reference 5/ Standard/DAD/ MS	12.7 ± 0.4	10.4 ± 0.2	0.038
11	25.6	282	567	535(23),493(49),387(32),285(25),197(13)	Caffeic acid derivative	DAD/MS	2.3 ± 0.1	1.68 ± 0.05	0.001
12	27.5	290,326sh	537	493(50),359(17),295(33),179(75),135(100)	Lithospermic acid A	Reference 5/ DAD/MS	2.25 ± 0.01	1.9 ± 0.1	0.999
13	30.6	288,334sh	287	151(35),135(100)	Eriodictyol	Reference 5/ Standard/DAD/ MS	0.87 ± 0.01	0.53 ± 0.04	0.001
					Total phenolic acids		48 ± 1	47 ± 1	0.251
					Total flavonoids		19.5 ± 0.3	19.0 ± 0.1	0.010
					Total phenolic compounds		67 ± 1	66 ± 1	0.097

References: (1) Hossain et al. (2010); (2) Nagy et al. (2011); (3) Vergara-Salinas et al. (2012); (4) Vallverdú-Queralt et al. (2014); (5) Martins et al. (2015).

time. The presence of salvianolic acid I with the same molecular weight was reported in thyme by Dapkevicius et al. (2002) and Nagy, Solar, Sontag, and Koenig (2011), although no sufficient elements for assigning that identity to the compound detected herein, so that the compound was just identified as a caffeic acid trimer. Compound 11 ($[M-H]^-$ at *m/z* 567) should also correspond to a caffeic acid derivative, owing to its UV spectrum and the observation of an MS^2 fragments at *m/z* 493, coherent with salvianolic acid A, furthermore, the fragment at *m/z* 197 could be attributed to dihydroxyphenyl-lactic acid (danshensu); however, no definite structure could be matched for the compound, so that it remains as an unidentified caffeic acid derivative. The presence of caffeic acid

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 peppermint extracts prepared from non-irradiated and irradiated samples (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Type of identification	Quantification (mg/g)		*t*-Students test *p*-value
							0 kGy	10 kGy	
1′	5.1	328	353	191(100),179(27),173(5),161(15),135(30)	3-*O*-Caffeoylquinic acid	Reference 1/DAD/MS	0.87 ± 0.02	0.76 ± 0.01	0.001
2′	7.1	328	353	191(100),179(90),173(50),161(20),135(57)	5-*O*-Caffeoylquinic acid	References 2,3/ Standard/DAD/MS	1.4 ± 0.1	1.2 ± 0.1	0.025
3′	11.1	326	179	135(100)	Caffeic acid	References 2,3,4,5/ Standard/DAD/MS	0.44 ± 0.01	0.5 ± 0.1	0.134
4′	14.5	348	637	285(100)	Luteolin-*O*-diglucuronide	References 2,3,7/DAD/ MS	7.1 ± 0.2	6.22 ± 0.01	0.001
5′	15.7	288,330sh	537	493(45),313(18),295(36),269(55),197(36),179(64),135(100)	Caffeic acid trimer	DAD/MS	3.1 ± 0.2	2.9 ± 0.2	0.115
6′	16.1	284,332sh	595	287(100)	Eriodictyol-*O*-rutinoside	References 3,7/DAD/MS	100 ± 1	102.47 ± 0.01	0.005
7′	17.1	286,336sh	449	287(100)	Eriodictyol-*O*-hexoside	DAD/MS	2.2 ± 0.2	2.1 ± 0.1	0.157
8′	19.0	350	593	285(100)	Luteolin-7-*O*-rutinoside	References 2,3,6/ Standard/DAD/MS	30.2 ± 0.1	30.3 ± 0.6	0.677
9′	19.8	348	461	285(100)	Luteolin-7-*O*-glucuronide	References 2,3,7/DAD/ MS	11.2 ± 0.2	10.0 ± 0.4	0.002
10′	20.0	282,330sh	579	271(100)	Naringenin-*O*-rutinoside	References 2,3/DAD/MS	3.1 ± 0.1	3.0 ± 0.2	0.362
11′	21.5	278,338sh	717	537(34),519(50),493(39),339(29),321(37),313(6),295(100),197(3),179(11),161(5),135(11)	Salvianolic acid B/E/L	References 2,3/DAD/MS	13 ± 1	13.4 ± 0.3	0.276
12′	22.6	286,338sh	609	301(100)	Hesperetin-*O*-rutinoside	DAD/MS	5.5 ± 0.3	5.6 ± 0.2	0.302
13′	23.6	330	359	197(13),179(20),161(100),135(21)	Rosmarinic acid	References 2,3,4,5/ Standard/DAD/MS	25 ± 1	25.1 ± 0.1	0.291
14′	24.0	288,340sh	493	313(5),295(100),279(3),197(14),179(8),135(5)	Salvianolic acid A	DAD/MS	10.3 ± 0.5	9.72 ± 0.01	0.065
					Total phenolic acids		53 ± 3	54 ± 1	0.939
					Total flavonoids		159 ± 2	159.7 ± 0.1	0.248
					Total phenolic compounds		212 ± 4	213.7 ± 0.5	0.607

References: (1) Clifford et al. (2003); (2) Kapp et al. (2013); (3) Riachi and De Maria (2015); (4) Pérez et al. (2014); (5) Lv et al. (2012); (6) Areias et al. (2001); (7) Krzyzanowska et al. (2011).

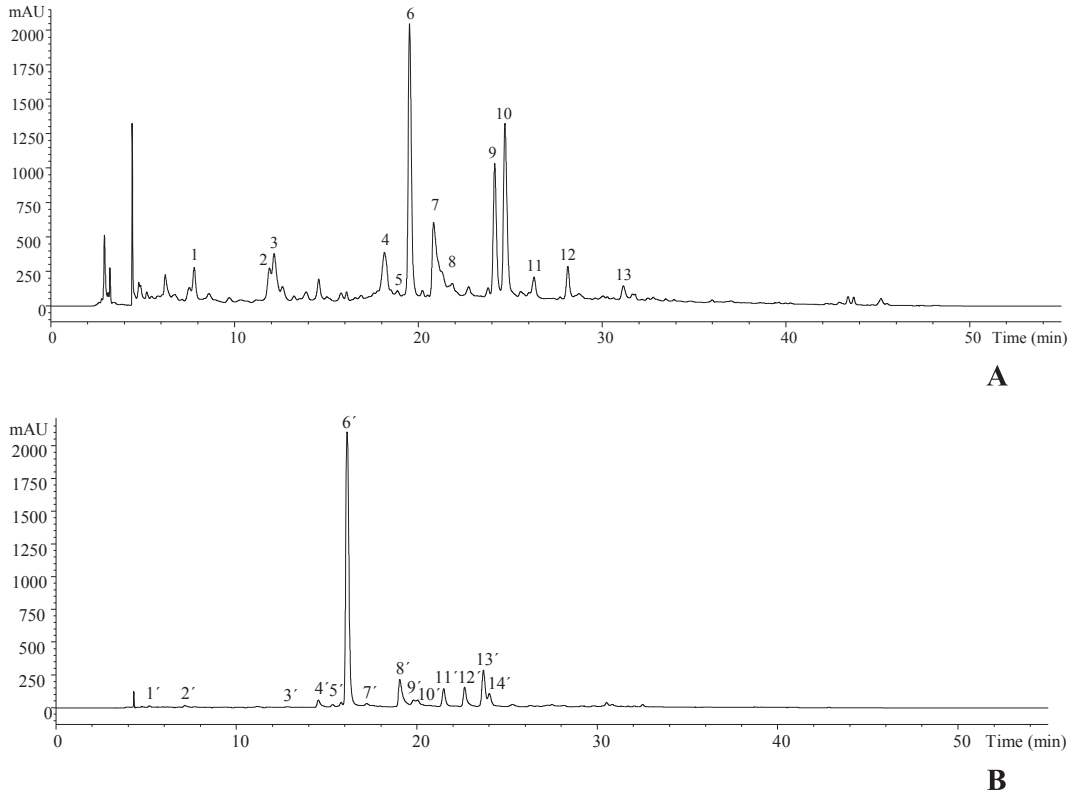

Fig. 1. Individual profile of thyme (A) and peppermint (B) irradiated with 10 kGy recorded at 280.

hexoside has been already reported in thyme by Hossain et al. (2010), Nagy et al. (2011), Vergara-Salinas et al. (2012) and Vallverdú-Queralt et al. (2014). To the best of our knowledge the other three compounds tentatively identified herein have not been previously cited in *T. vulgaris*.

Regarding peppermint, caffeic acid (compound 3′), chlorogenic acid (i.e., 5-*O*-caffeoylquinic acid; compound 2′), luteolin-7-*O*-rutinoside (compound 8′) and rosmarinic acid (compound 13′) were positively identified according to their retention, mass spectra and UV–vis characteristics in comparison with commercial standards. These compounds were also described in *Mentha piperita* leaves (using petroleum ether, chloroform, ethyl ether, ethyl acetate, acetone, methanol, ethanol, ethanol 80% and ethanol 30% and a boiling water extract) of commercial and non-commercial samples (Areias, Valentão, Andrade, Ferreres, & Seabra, 2001), in *M. piperita* infusions (Kapp et al., 2013; Pérez, Rocha-Guzmán, Mercado-Silva, Loarca-Piña, & Reynoso-Camacho, 2014), in extracts from conventional and organically grown peppermint samples (soluble free phenolics, soluble conjugated phenolics, insoluble bound phenolics) (Lv et al., 2012) and in a revision of the literature performed by Riachi and De Maria (2015).

Compound 1′ was identified as 3-*O*-caffeoylquinic acid based on its MS^2 fragmentation, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity of 72% relative to the base peak, considered characteristic of 3-acylchlorogenic acids as reported by Clifford, Johnston, Knight, and Kuhnert (2003). The sample also presented other caffeic acid derivatives, namely compounds 5′, 11′ and 14′. The pseudomolecular ion ($[M-H]^-$ at *m/z* 537) and fragmentation pattern of peak 5′ were consistent with a caffeic acid trimer, although it eluted at an earlier retention time than lithospermic acid A. As above discussed for peak 9 in the thyme sample (Table 2), besides lithospermic acid A, the molecular weight of the compound would also match that of salvianolic acid H/I, reported by Kapp et al. (2013) in peppermint teas, although no further support for that identity could be obtained, so that in our case the compound has been just assigned as a caffeic acid trimer. The molecular weight of compound 14′ ($[M-H]^-$ at *m/z* 493, compound 14′) might correspond to salvianolic acid A, whereas that of compound 11′ ($[M-H]^-$ at *m/z* 717) might correspond to salvianolic acids B or E, whose presence was reported in peppermint teas by Kapp et al. (2013), or salvianolic acid L, cited by Krzyzanowska, Janda, Pecio, Stochmal, and Oleszek (2011) in the aerial parts of *Mentha* species. Similar caffeoyl derivatives have been cited in mentha samples by other authors as reviewed by Riachi and De Maria (2015). All these compounds were quantified based on caffeic and rosmarinic acid calibration curves.

The remaining compounds were identified as flavonoids. Peaks 4′ ($[M-H]^-$ at *m/z* 637) and 9′ ($[M-H]^-$ at *m/z* 461) were assigned as luteolin glycosides, based on their UV spectra (λ_{max} around 350 nm) and the production of an MS^2 fragment ion at *m/z* 285. Compound 9′ can be assumed as luteolin-7-*O*-glucuronide by comparison with a commercial standard, whereas compound 4′ was tentatively assigned as luteolin-*O*-diglucuronide. The presence of luteolin glucuronides in *M. piperita* samples was also reported by Krzyzanowska et al. (2011), Kapp et al. (2013) and Riachi and De Maria (2015). Compounds 6′ ($[M-H]^-$ at *m/z* 595) and 7′ ($[M-H]^-$ at *m/z* 449) were tentatively identified as eriodictyol-*O*-rutinoside and eriodictyol-*O*-hexoside, respectively, previously described in *M. piperita* by Krzyzanowska et al. (2011) and Riachi and De Maria (2015). Based on the mass spectra, compounds 10′ ($[M-H]^-$ at *m/z* 579) and 12′ ($[M-H]^-$ at *m/z* 609) were identified as rutinosyl derivatives of the flavanones naringenin and hesperetin, respectively. The presence of narirutin (i.e., naringenin-7-*O*-rutinoside) in peppermint samples was cited by Kapp et al. (2013) and Riachi and De Maria (2015).

In a previous study rosmarinic acid and luteolin-7-*O*-

glucuronide were found as the most abundant compounds in thyme (Martins et al., 2015). These compounds were also relevant components in the sample analysed herein, although in this case rosmarinic acid hexoside appeared as the most abundant phenolic compound. These differences could be related with the natural variability inherent to plants grown under different environmental conditions that influence their secondary metabolism (Riachi & De Maria, 2015). Eriodictyol-7-*O*-rutinoside was the most abundant compound in peppermint, in agreement with a previous study performed by Areias et al. (2001). The irradiation at 10 kGy did not affect the phenolic composition in both plant samples in relation to non irradiated control samples, which could be explained by the reduced water activity of this matrix. The molecules preservation by irradiation mostly depends on the food composition in water content, temperature and the presence or absence of oxygen in the process, thus the dried food has a reduced effect of radiolytic products production (Sadecká, 2007). However, a different effect was observed in a study performed by Pereira et al. (2015b), where the irradiated sample at 10 kGy showed the highest content in phenolic compounds. This could be explained by the usage of a high dose of radiation that leads to an increase in the extractability of certain phenolic compounds. Another reason that could justify this effect is the water activity that remains after the drying process. Thereby, *Ginkgo biloba* sample might have higher water content, which triggered a higher formation of radiolytic compounds, leading to the changes verified in the phenolic contents (Tezotto-Uliana, Silva, Kluge, & Spoto, 2015). Therefore, it can be concluded that gamma irradiation does not conduct to a linear behaviour towards the conservation of compounds in plants, and depends also on other factors (e.g., water composition, different compounds present in plants, dose applied). Nonetheless, this radiation dose could be recommended as adequate to decontaminate these plants without affecting their contents on phenolic compounds. The same dose was also recommended by Machhour, Hadrami, Imziln, Mouhib, and Mahrouz (2011) with similar purposes.

In order to correlate the sample's cytotoxic effects with the phenolic composition, correlation factors were obtained between total phenolic acids and total flavonoids, and the GI_{50} values obtained for the four cell lines. The results showed high correlations in both plant samples for three of the cell lines, with the exception of MCF-7, where no correlation was found between the total contents of these phenolic groups. Nevertheless, the thyme cytotoxic activity obtained in MCF-7 cell line was highly correlated with caffeic acid ($R^2 = 0.7100$), caffeic acid trimer ($R^2 = 0.7709$), methyleriodictyol-*O*-pentosylhexoside ($R^2 = 0.4375$), rosmarinic acid hexoside ($R^2 = 0.4247$), luteolin-7-*O*-glucoside ($R^2 = 0.4305$), and lithospermic acid A ($R^2 = 0.4272$), while in peppermint the main contributor compounds were caffeic acid ($R^2 = 0.8586$), caffeic acid trimer ($R^2 = 0.7667$), luteolin-7-*O*-rutinoside ($R^2 = 0.6649$) and luteolin-7-*O*-glucuronide ($R^2 = 0.6466$).

The cytotoxic effects of thyme extracts for the other three cell lines, also presented high correlation factors, being NCI-H460 cell line highly correlated with total flavonoids ($R^2 = 0.9991$) and HeLa and HepG2 cell lines with total phenolic acids ($R^2 = 0.7483$ and 0.8139, respectively). The individual flavonoids that were highly correlated with NCI-H460 cell line were apigenin 6,8-di-C-glucoside ($R^2 = 0.8294$), methyleriodictyol-*O*-pentosylhexoside ($R^2 = 0.7416$), luteolin-7-O-glucoside ($R^2 = 0.7708$) and eriodictyol ($R^2 = 0.9235$), while the phenolic acids were caffeic acid hexoside ($R^2 = 0.9226$, for HeLA), caffeic acid ($R^2 = 0.5037$, for HeLA), rosmarinic acid hexoside ($R^2 = 0.7211$ and 0.5939, for HeLA and HepG2, respectively), rosmarinic acid ($R^2 = 0.5748$, for HepG2) and caffeic acid trimer ($R^2 = 0.8894$ and 0.5696, for HeLA and HepG2, respectively). In relation to peppermint extracts an opposite effect was observed in relation to thyme, being NCI-H460 cell line correlated with the total phenolic acids ($R^2 = 0.5319$), while HeLa and HepG2 cell lines were correlated with total flavonoids ($R^2 = 0.6946$ and 0.6214, respectively). The individual compounds that were responsible for these correlations were caffeic acid trimer ($R^2 = 0.5246$, for NCI-H460), rosmarinic acid ($R^2 = 0.5917$, for NCI-H460), luteolin-*O*-diglucuronide ($R^2 = 0.4148$ and 0.6462, for HeLA and HepG2, respectively), eriodictyol-*O*-rutinoside ($R^2 = 0.4075$ and 0.6986, for HeLA and HepG2, respectively), luteolin-7-*O*-rutinoside ($R^2 = 0.6409$ and 0.5638, for HeLA and HepG2, respectively) and luteolin-7-*O*-glucuronide ($R^2 = 0.4207$ and 0.7956, for HeLA and HepG2, respectively). Most of the mentioned phenolic compounds were not the main molecules present in the sample, meaning that synergistic effects are probably observed between the compounds, in order to enhance these activities.

Overall, all samples showed cytotoxic properties in human tumor cell lines, but with no toxicity for non-tumor cells. The different irradiation doses did not affect these properties or the phenolic composition of the peppermint samples. However, thyme samples irradiated at 10 kGy showed higher cytotoxicity for tumor cell lines in comparison with the other doses applied. Therefore, for the studied species, it is confirmed that 10 kGy can be applied because it did not affect the bioactive properties of these plants. The studied plants can represent a rich source of antioxidant compounds of phenolic nature: flavonols, flavones, flavanones and phenolic acid derivatives, which might contribute to the prevention and control of diseases through their incorporation into a normal diet or as supplements. Therefore, irradiation can be considered as a decontamination and preservation process (as described and tested by other authors), because when analysing the influence of this technology in compounds that provide the bioactive potential, it was found that it can be applied up to the maximum dose recommended in legislation (10 kGy) because it does not affect their concentration. This technology represents an added-value solution to meet the requirements of the food and pharmaceutical industries in the acquisition of high quality raw materials.

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