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Effects of γ-Irradiation of Wild Thyme (*Thymus serpyllum* L.) on the Phenolic Compounds Profile of Its Ethanolic Extract

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The presented study revealed that there were changes in the phenolic compounds profile of extract of wild thyme (*Thymus serpyllum* L.) after γ-irradiation at the dose of 5 kGy. Ethanolic extracts of irradiated and non-irradiated herb were prepared and their compounds were analyzed by RP-HPLC-DAD technique. Between thirty two detected constituents, twelve phenolic compounds classified as hydroxybenzoic and hydroxycinnamic acids derivatives, flavones and flavanones were identified. Among them, caffeic acid derivatives and flavones predominated with the highest content of rosmarinic acid and luteolin-7-*O*-glucoside, respectively. Additionally, thymol was recognized in the analyzed extracts. γ-Irradiation slightly affected the quantitative profile of phenolic compounds of a wild thyme ethanolic extract. Only four constituents differed significantly ($P<0.05$) in terms of their content in the irradiated and non-irradiated samples. The content of phenolic acids (*p*-coumaric and caffeic acids) decreased and that of flavonoid aglycons (luteolin and eriodictyol) increased after the γ-ray treatment.

INTRODUCTION

The genus *Thymus*, commonly known as thyme, is wildly distributed in the Old Word [Morales, 2002]. It consists of about 215 species, among which *Thymus vulgaris* L. is most common and commercially used. In turn *Thymus serpyllum* L., well known as wild thyme, is widespread in the North of Europe.

Thyme is an aromatic plant widely used for both nutritional and medicinal purposes since ancient times. This herb added to dishes and foodstuff enhances or improves food flavor and also, because of its antioxidant and antibacterial potential, acts as a preservative agent [Viuda-Martos *et al.*, 2010; Jayasena & Jo, 2014]. These effects are ascribed to secondary metabolites present in *Thymus* species. As the aromatic plant, thyme is rich in essential oils including mainly oxygenated monoterpenes [Nikolić *et al.*, 2014; Sonmezdag *et al.*, 2016]. A broad spectrum of phenolic compounds of thyme was identified, too [Fecka & Turek, 2007; Boros *et al.*, 2010; Miron *et al.*, 2011]. A majority of these constituents are phenolic acids and flavonoids as well as their glycosides and other derivatives. A high content of bioactive compounds is also associated with properties of herbs being beneficial to human health [Viuda-Martos *et al.*, 2010]. Both essential oils and phenolic compounds of *Thymus* species are characterized by the mentioned strong antioxidant and antibacterial activities [Amarowicz *et al.,* 2009; Nikolić *et al.,* 2014; Martins *et al.,* 2015]. In addition, they were reported to exert anti-inflammatory, antiviral, antitumor, and digestion-stimulating effects [Viuda-Martos *et al.*, 2010; Nikolić *et al.,* 2014; Opara & Chohan, 2014]. In traditional medicine, decoctions, infusions (herbal teas) and hydroalcoholic extracts are obtained from thyme herbs [Martins *et al.,* 2015] and used as spasmolytic, antiseptic, antitussive, and expectorant preparations [Rasooli & Mirmostafa, 2002].

Consumer and industrial use of dry herbs and spices requires their appropriate microbiological quality. Most of them are dried in the open air and can become seriously contaminated by air- and soil-borne bacteria, fungi and insects [Sadecka, 2007]. Non-thermal technologies of food preservation are currently developed [Mañas & Pagán, 2005; Tokuşoğlu, 2016]. They extend the shelf-life of food products by microbial inactivation without the addition of preservatives. Moreover, sensory, nutritional and functional properties of foods are maintained or are changed to a small extent.

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Among these food-preserving methods there are technologies based on the process of food exposure to ionizing irradiation, the source of which could be γ-rays (from radioisotopes ^{60}Co or ^{137}Cs), X-rays or electron beams [Kilcast, 1995; Mañas & Pagán, 2005]. During food γ-irradiation treatment, reactive species are generated as a result of one electron removal from a water molecule. Interactions between the generated free radicals and DNA of microorganisms underlie the process that leads to cell death and food decontamination [Kilcast, 1995]. Moreover, in the direct action of γ-rays, absorption of radiation energy by the target molecules causes death not only to microorganisms, but also to parasites and insects [Mañas & Pagán, 2005; Sadecka, 2007].

γ-Irradiation is currently successfully used for decontamination of dried herbs and spices [Sadecka, 2007], but its effects on their bioactive compounds, being crucial for their culinary and medical applications, are not fully elucidated. Literature works have reported on both an increase and a decrease in contents of total phenolics, total flavonoids and individual phenolic compounds, as well as on the antioxidant activity of the irradiated compared to the non-irradiated herbs [Suhaj *et al.*, 2006; Pérez *et al.*, 2007; Pereira *et al.*, 2016a; Janiak *et al.*, 2017]. Other studies showed a minimal or insignificant influence of γ-irradiation on contents of phenolic compounds and antioxidant activity of spices and herbs [Brandstetter *et al.*, 2009; Nagy *et al.*, 2011; Pereira *et al.*, 2016b]. The aim of our study was, therefore, to determine whether γ-irradiation of wild thyme is the cause of qualitative and quantitative changes in the phenolic compounds profile of its ethanolic extract. To the best of our knowledge, the influence of γ-irradiation on contents of individual phenolic compounds of *Thymus serpyllum* L. has not been investigated so far.

MATERIAL AND METHODS

Material and chemicals

Wild thyme (*Thymus serpyllum* L.) was obtained as herbal tea manufactured by the Bioprogramme Co. (Bulgaria), which has been working under IFS and ISO 9001:2008 standards. Herb used to *T. serpyllum* tea production was collected from the wild in Bulgaria and was certified IMO – Institute for Marketecology. Four boxes of tea, each containing 20×1 g bags were used in the study. Half of the boxes were irradiated and the rest of them were used to prepare control (non-irradiated) samples.

The HPLC standards: luteolin, luteolin-7-*O*-glucoside, apigenin and naringenin, were obtained from Extrasynthese (France). Thymol and acetonitrile of HPLC grade were acquired from Acros Organics (Belgium) and Merck (Germany), respectively. The other applied reference compounds, solvents and trifluoroacetic acid were purchased from Sigma-Aldrich (USA).

γ-Ray treatment

The boxes of wild thyme tea (in commercial packing) were irradiated at ^{60}Co source with the total activity of 8.2 kCi using a facility with a 4.0 L rotary irradiation chamber (ϕ 13.5 cm, H 22 cm). For the study of absorbed dose distribution, alanine film dosimeters (BioMax, Kodak, USA) were used, measured by e-scan ESR spectrometer (Bruker, USA) and calibrated in units of the absorbed dose in water. The average dose rate was 2.98 kGy/h. The minimum absorbed γ-ray dose and dose uniformity ratio (D_{max}/D_{min}) were 5±0.02 kGy and 1.25, respectively. The irradiated samples were stored at room temperature until further use (one month).

Preparation of extracts

Before extraction, wild thyme was removed from tea bags. The 6×3.5 g portions of thyme (3 irradiated and 3 non-irradiated) were weighed, suspended in 95% ethanol at a solid to solvent ratio of 1:20 (v/v) and shaken (OS-10 Orbital Shaker, Biosan, Latvia) at room temperature for 24 h [Amarowicz *et al.*, 1999]. Afterwards, the mixtures were filtrated through Whatman Grade 1 paper. The residues were extracted again under the same conditions using additional 70 mL of 95% ethanol. Solvent was evaporated from the combined filtrates under vacuum (Rotavapor R-200, Büchi Labortechnik, Switzerland) and the remaining water was removed by lyophilization at -70°C and 0.013 mbar for ~48 h using a Labconco freeze dryer system (Lyph Lock 6, Labconco, USA). Mass balance was performed to calculate % recovery of crude phenolic extracts.

UV-Vis spectra

UV-Vis absorption spectra of the methanolic solutions of extracts of the irradiated and non-irradiated wild thyme were measured with a Beckman DU-7500 spectrophotometer (Beckman Instruments, USA) by scanning over a wavelength range from 220 to 500 nm. The first and second derivatives of spectra were calculated and plotted using software of the Beckman DU-7500 spectrophotometer.

Analysis of phenolic compounds

The HPLC Shimadzu system (Shimadzu, Japan) consisting of CBM-20A communications bus module, DGU-20A_{5R} degassing unit, two LC-30AD pumps, SIL-30AC autosampler, SPD-M30A diode array detector, RF-20A_{XS} fluorescence detector, and CTO-20AC column oven was used to analyze profiles of the phenolic compounds of the extracts. Samples were dissolved in 80% (v/v) methanol (10 mg/mL) and 10 μL of solutions were injected into a pre-packed Luna C8(2) column (4.6×150 mm, particle size 3 μm, Phenomenex, USA). Separation was carried out at 25°C with 1 mL/min flow rate of the mobile phase which consisted of solvent A: acetonitrile-water-trifluoroacetic acid (5:95:0.1, v/v/v) and solvent B: acetonitrile-trifluoroacetic acid (100:0.1, v/v). The gradient elution system was developed and used according to the following program: 0–10 min, 0–28% B; 10–13 min, 28–60% B; 13–16.5 min, 60–85% B; 16.5–18 min, 85–0% B and 18––22 min 0% B. The scans of chromatographic eluent passing through the diode array detector were recorded in a wavelength range from 200 to 600 nm [Karamać *et al.*, 2015]. The fluorescence detector was set at excitation and emission wavelengths of 276 and 310 nm, respectively [Cussonneau *et al.*, 2007].

Phenolic compounds were identified by comparison of retention times and absorption maxima of UV-Vis spectra

of the compounds with those of the corresponding standards. Individual, identified compounds were quantified under calibration curves of the corresponding standards. The peak areas per gram of extract were determined for compounds with not fully recognized structure.

Statistical analysis

The content of individual phenolic compounds was determined for three extracts of wild thyme prepared in parallel and results were presented as means ± standard deviations. The analysis of variance and comparison of treatment means by Student's *t*-test ($p<0.05$) were carried out. The statistical analysis was performed using GraphPad Prism software (version 6.04 for Windows, GraphPad Software, USA).

RESULTS AND DISCUSSION

Legislation of the European Union permits the use of γ-ray treatment of dried aromatic herbs, spices, and vegetable seasonings for microbiological decontamination with maximum average absorbed dose of 10 kGy of [EC, 1999]. In the present study, the mode of γ-irradiation processing of the wild thyme tea with the dose of 5 kGy was chosen on the basis of preliminary microbiological test and considering the type of herb packaging. The selected γ-irradiation dose was effective to reduce the total mesophilic bacteria count by four log cycles and achieve 2.78 log CFU/g (unpublished data), which is below the value acceptable for herbs as specified by the World Health Organization [WHO, 1998]. Some previous study showed that γ-irradiation at dose of 5 kGy was sufficient to eliminate or reduce up to an acceptable level the microbiological contamination of herbs, spices or other dried food ingredients [de Camargo *et al.*, 2015; Deng *et al.*, 2015; Napoli *et al.*, 2016].

UV-Vis absorption spectra of ethanolic extracts of wild thyme before and after γ-irradiation were shown in Figure 1A. The spectrum of the untreated sample was characterized by three absorption maxima at wavelengths of 282, 326 and 409 nm. The first two of these indicated the presence of phenolic compounds in the extracts. Hydroxybenzoic acid derivatives have their absorption maxima between 200 and 290 nm [Robbins, 2003]. Also all subclasses of flavonoids and tannins (proanthocyanidins and hydrolysable tannins) exhibit λ_{max} at around 240–290 nm [de Rijke *et al.*, 2006; Lin & Harnly, 2012]. This band of spectrum is due to the benzoyl structure of phenolic compounds or conjugation of ring A of flavonoids and its substitution pattern. In turn, the absorption maximum around 305–360 nm is typical of hydroxycinnamic acid derivatives and flavonoids of two subclasses: flavones and flavonols [Robbins, 2003; de Rijke *et al.*, 2006; Karamać *et al.*, 2015]. It is affected by an additional propene group in the structure and conjugation of B and C rings *via* a double bond, respectively. Our previous research [Janiak *et al*., 2017] demonstrated that wild thyme ethanolic extract did not contain proanthocyanidins, hence in current experiment, maxima of UV-Vis spectrum of extract (Figure 1A) were possibly caused by the presence of phenolic acids and/or flavonoids. The UV-Vis spectrum of the extract of γ-irradiated thyme possessed maxima exactly at the same wavelengths. The maxima of the first and second derivatives of absorbance of spectra of the γ-ray-treated samples were not shifted compared to the control (Figures 1B and C). This indicated only small differences in phenolic compounds profiles of both extracts.

The HPLC-DAD analysis was performed to accurately identify and quantify phenolic compounds of the wild thyme ethanolic extracts. Results of separations of the non-irradiated and irradiated samples were shown in Figure 2 and Table 1. The qualitative profile of phenolic compounds of *T. serpyllus* was not changed after γ-irradiation at the dose of 5 kGy, *i.e.* 32 peaks were recorded on chromatograms at 280 nm for each of the extracts. Some of the numbered peaks were not visible when absorbance was read at 320 nm. Compounds corresponding to these peaks (number 2, 3 and 9) were recognized as hydroxybenzoic acid derivatives: gallic, protocatechuic and syringic acids, respectively. Their content in thyme extracts was low and ranged from 0.58 to 1.56 mg/g (Table 1). These three phenolic acids have been identified in wild thyme in recent studies [Miron *et al.*, 2011; Sonmezdag *et al.*, 2016]. Sonmezdag *et al.* [2016] determined protocatechiuc acid in a higher amount – 4.82 mg/g herb. On the other hand, Fecka & Turek [2008] and Boros *et al.* [2010] did not find hydroxybenzoic acid derivatives in *T. serpyllus*. The last peak (32) which was noticeable only on chromatogram at 280 nm (Figure 2) corresponded to

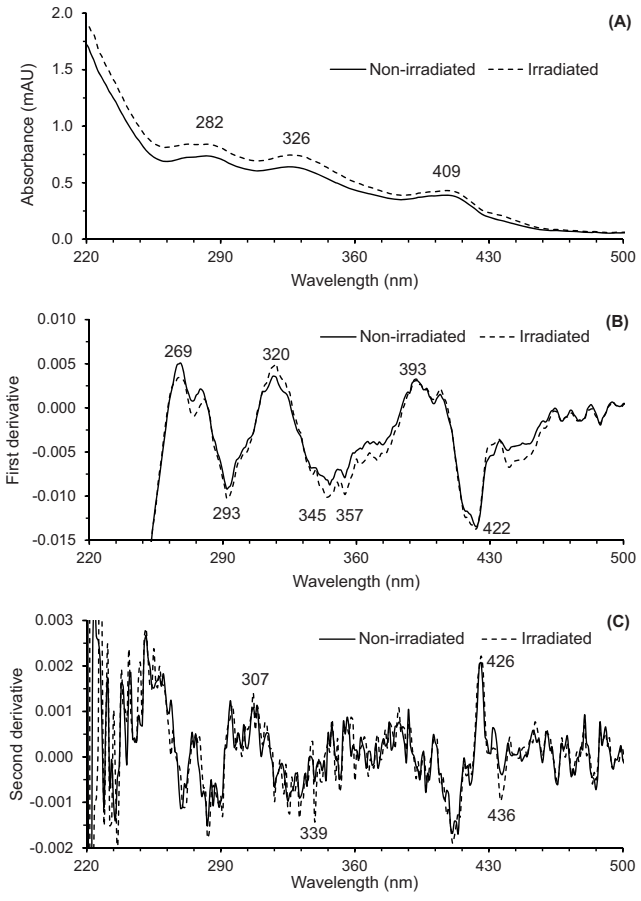

FIGURE 1. UV-Vis spectra of extracts of irradiated and non-irradiated wild thyme **(A)** and their first **(B)** and second **(C)** derivatives.

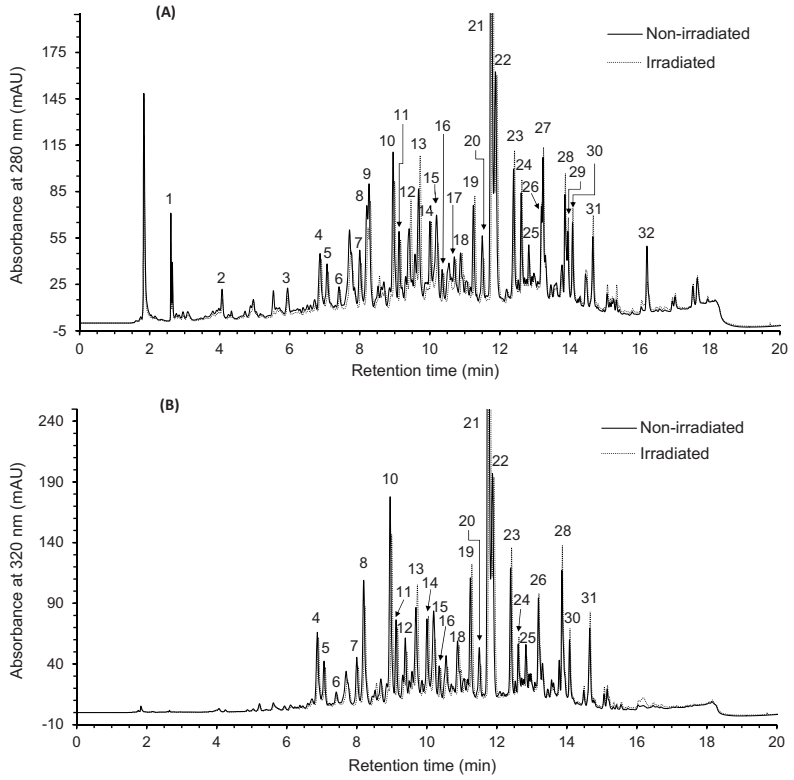

FIGURE 2. HPLC chromatograms of phenolic compounds of extracts of irradiated and non-irradiated wild thyme detected at 320 nm **(A)** and 280 nm **(B)**.

thymol. Its presence in ethanolic extracts was confirmed on the basis of a chromatogram recorded using a fluorescence detector at excitation and emission wavelengths of 276 and 310 nm, respectively (Figure 3). Thymol, which in terms of structure is a monoterpene phenol, was determined as the main compound of essential oils of *T. serpyllus* [Nikolić *et al.*, 2014] with the content of 1702 mg/100 g dry herb [Sonmezdag *et al.*, 2016]. Thymol was also extracted in our study, because of its good solubility in ethanol, but its content in the extract was not very high (Table 1). Probably,

TABLE 1. Compounds identified and quantified in extracts of irradiated and non-irradiated wild thyme*.

Peak No.	Identified compound	t_R (min)	λ_{max} (nm)	Content (mg/g extract)	
				Non-irradiated	Irradiated
2	Gallic acid	4.03	272	0.58±0.102[a]	0.43±0.048[a]
3	Protocatechuic acid	5.90	260, 294	1.03±0.176[a]	0.83±0.098[a]
4	Chlorogenic acid	6.83	301sh, 326	2.24±0.402[a]	1.90±0.227[a]
8	Caffeic acid	8.16	301sh, 323	1.27±0.196[a]	0.93±0.025[b]
9	Syringic acid	8.22	275	1.56±0.324[a]	1.40±0.202[a]
13	Luteolin-7-*O*-glucoside	9.64	268, 338	4.09±0.712[a]	4.21±0.443[a]
15	*p*-Coumaric acid	10.15	296sh, 311	1.17±0.180[a]	0.87±0.091[b]
21	Rosmarinic acid	11.70	301sh, 329	12.5±2.63[a]	11.5±1.51[a]
26	Luteolin	13.16	253, 347	1.55±0.440[b]	2.43±0.140[a]
27	Eriodictyol	13.21	288	0.65±0.198[b]	1.09±0.065[a]
28	Apigenin	13.84	267, 336	0.99±0.099[a]	1.16±0.078[a]
29	Naringenin	13.93	288	0.74±0.106[a]	0.82±0.052[a]
32	Thymol	16.19	202, 221, 277	4.92±1.651[a]	3.78±0.561[a]

*Data are expressed as the mean±standard deviation (n=3); values in the same row having different letters differ significantly ($P<0.05$).

part of extracted thymol volatilized during extract drying (evaporation of solvent and lyophilization).

Compounds 4, 8, 10, 15, 18, 20–23 and 25 were characterized by the UV-DAD spectra with maximum absorption at 311–329 nm and shoulders at the short wavelength and were classified as hydroxycinnamic acid derivatives. Four of them were identified as: chlorogenic (4), caffeic (8), *p*-coumaric (15) and rosmarinic (21) acids. Their content in the ethanolic extracts was in a decreasing order of: rosmarinic acid >> chlorogenic acid > caffeic acid > *p*-coumaric acid (Table 1). Rosmarinic acid was present in about 3–30 times higher amount than that of other identified phenolic compounds. The dominant content of rosmarinic acid in *T. serpyllus* and the presence of other identified hydroxycinnamic acid derivatives corresponded to literature data [Kulišić *et al.*, 2006; Boros *et al.*, 2010; Miron *et al.*, 2011]. Sonmezdag *et al.* [2016] additionally determined ferulic acid and rosmarinic acid glucoside among hydroxycinnamic acid derivatives of wild thyme. In turn, Fecka & Turek [2008] and Miron *et al.* [2011] detected lithospermic acid and caffeic acid ethyl ester, respectively.

Flavonoids belonging to subclasses of flavones and flavanones were found in the analyzed wild thyme ethanolic extracts. Luteolin-7-*O*-glucoside (13), luteolin (26) and apigenin (28) were recognized as compounds form the first subclass (Figure 2, Table 1). Based on UV-DAD spectra (data not shown), also some non-identified compounds (5, 7, 11, 14, 16, 19) could be included to flavones. Because retention times of peaks corresponding these compounds were shorter than t_R of luteolin and apigenin, the compounds with not fully identified structure were probably sugar derivatives of flavones. We did not find apigenin-7-*O*-glucoside and apigenin-6,8-di-*C*-glucoside among them, although they were noted in previous studies [Kulišić *et al.*, 2006; Boros *et al.*, 2010; Sonmezdag *et al.*, 2016]. Whereas a few other glucoside, glucuronide and rutinoside derivatives of luteolin and apigenin of wild thyme were described in literature [Fecka & Turek, 2008; Miron *et al.*, 2011; Sonmezdag *et al.*, 2016]. Flavanone aglycons: eriodictyol, eriocitrin and naringenin and their glycosides: eriodictyol-7-*O*-glucuronide and naringin, have so far been determined in wild thyme [Fecka & Turek, 2008; Boros *et al.*, 2010; Miron *et al.*, 2011; Sonmezdag *et al.*, 2016]. In the current study, *T. serpyllus* ethanolic extracts contained eriodictyol (27) and naringenin (29) (Table 1, Figure 2), but eriocitrin and naringin were absent.

The results of comparison of contents of individual phenolic compounds identified in the ethanolic extracts of the irradiated and non-irradiated wild thyme were present in Table 1. Figure 4 shows HPLC peak areas of the corresponding com-

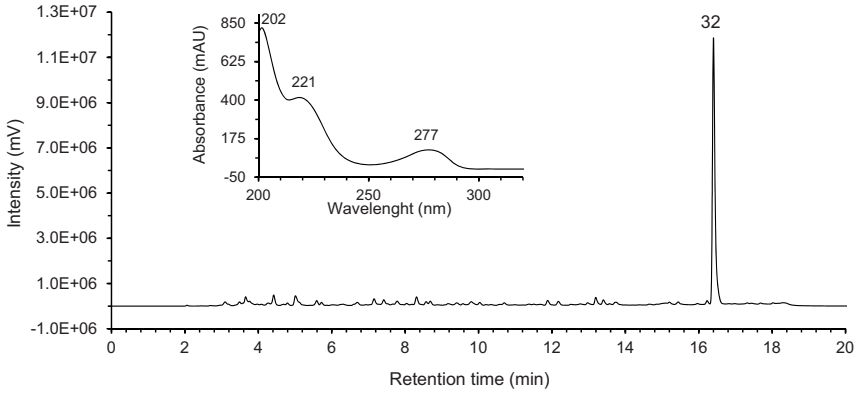

FIGURE 3. HPLC chromatogram of an extract of wild thyme recorded using fluorescence detector with UV-Vis spectrum corresponding peak 32 identified as thymol.

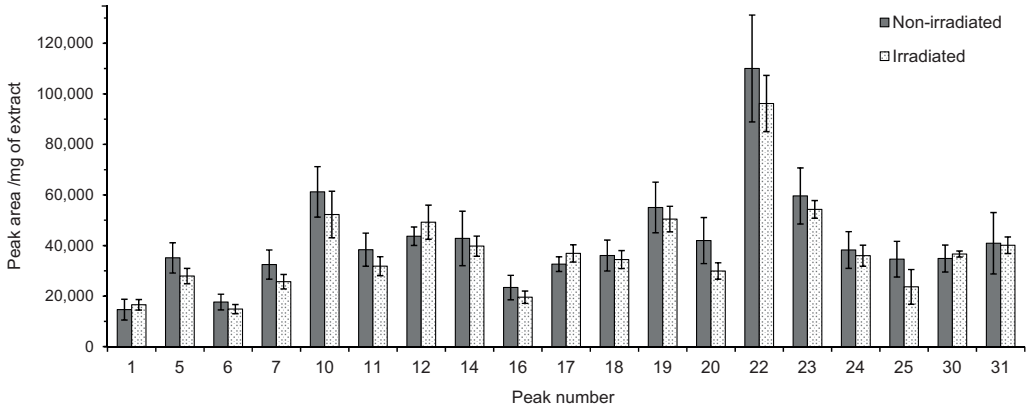

FIGURE 4. HPLC peak area for compounds with not fully identified structure detected in extracts of irradiated and non-irradiated wild thyme. Peak numbers are the same as those in chromatograms in Figure 1. Data are expressed as the mean (n=3) with ± standard deviation (error bars). Values do not differ significantly (P>0.05) for any pair of results (irradiated and non-irradiated).

pounds with not fully identified structure detected in the γ–rays treated and control samples. Contents of all the constituents presented in Figure 4 and as many as nine of the identified compounds (Table 1) of extracts of the irradiated and non-irradiated *T. serpyllus* herb did not differ significantly ($P>0.05$). Thus, among thirty two considered compounds, the levels of only four were changed significantly after γ-irradiation at the dose of 5 kGy. The contents of *p*-coumaric and caffeic acids in the extracts decreased ($P<0.05$). But it should be noted that taking into account all other phenolic acids, completely and tentatively identified, there was a tendency for slight reduction in their content in the extract of thyme exposed to γ-radiation compared to the control (although changes were statistically insignificant). This remark agrees with results obtained for infusions from *Thymus vulgaris* L. [Pereira *et al.*, 2016a]. These authors showed a decrease in the contents of total phenolic acids as well as individual caffeic acid derivatives after irradiation at 10 kGy and supposed that phenolic acids were degraded to some extent because of their lower stability against γ-irradiation. Additionally, Nagy *et al.* [2011] noticed no release of caffeic acid from rosmarinic acid and from other caffeic acid derivatives on account of action of γ-rays on dried oregano, sage and common thyme. Our study confirmed these observations. In turn, a small but statistically significant increase was reported in luteolin and eriodictyol contents of the ethanolic extracts of the irradiated and non-irradiated wild thyme (Table 1). Higher contents of total and some individual flavonoids in both extracts and infusions of irradiated dry plants compared to the non-irradiated controls were described in literature [de Camargo *et al.*, 2012; Pereira *et al.,* 2015; 2016a]. This phenomenon was explained by enhancement of compounds extractability caused by the γ-irradiation process. However, in the present study, statistically insignificant difference ($P>0.05$) in recovery of crude phenolic extract of irradiated and non-irradiated thyme (34.0 ± 0.45 and 31.1 ± 1.90, respectively) was noted. Because we observed the increase of content only flavonoid aglycons with trend to reduce the content of flavonoid glycosides (tentatively identified compounds 5, 7, 11, 14, 16, 19, changes statistically insignificant), so it can be assumed that a few glycoside bonds were cleaved as a result of γ-irradiation. But this presumption should be verified in future research.

CONCLUSIONS

Ethanolic extract of wild thyme was a rich source of widely varied phenolic compounds mainly hydroxycynnamic acid derivatives and flavones. Among the identified phenolic compounds the highest contents were observed in the case of caffeic acid and its derivatives (rosmarinic and chlorogenic acids) as well as luteolin-7-*O*-glucoside and luteolin as flavones. Ethanol was able to extract also thymol from the wild thyme. The γ-irradiation at the dose of 5 kGy did not change the qualitative profile of phenolic compounds of the ethanolic extract obtained from *T. serpyllus* herb and affected slightly their quantitative composition. Between thirty two detected compounds, only two phenolic acids and two flavonoid aglycons differed significantly ($P<0.05$) in terms of their content in the irradiated and non-irradiated samples. It can be pointed out that the process of wild thyme exposure to γ-irradiation applied for its microbiological decontamination allows preserving the majority of bioactive compounds of phenolic nature present in herb.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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