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AKADÉMIAI KIADÓ

The effects of drying and fermentation on the bioaccessibility of phenolics and antioxidant capacity of *Thymus vulgaris* leaves

K. Ozkan*, A. Karadag and O. Sagdic

Food Engineering Department, Chemical and Metallurgical Engineering Faculty, Yildiz Technical University, 34210, Istanbul, Turkey

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ABSTRACT

Fresh thyme leaves (*Thymus vulgaris* L.) were dried at 45 °C for 5 h and naturally fermented at 20 °C in a brine solution containing salt and vinegar for 18 days. The ethanolic extracts of fresh (FT), dried (DT), and fermented-pickled (PT) thyme leaves were assessed in terms of total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity values and subjected to *in vitro* gastrointestinal digestion. TPC, TFC, and antioxidant capacity values of fermented thyme leaves were found significantly higher than of dried and fresh samples. The bioaccessibility index (BI) value for TPC and TFC was highest for PT and lowest for DT, indicating that both processes had different effects on the structure of phenolic compounds present in the thyme leaves. Similarly both Recovery and BI values of DPPH antioxidant capacity were highest for PT, but lowest for fresh samples. When CUPRAC assay was applied, the recovery % for FT and PT was similar, and the BI was higher for FT. Results showed that compared to the results of fresh thyme leaves, drying and pickling had a considerable effect on the initial phenolic compounds extracted and their fate during *in vitro* digestion.

KEYWORDS

thyme, pickling, food drying, digestion, antioxidant capacity

* Corresponding author. E-mail: kubra.ozkan@std.yildiz.edu.tr

1. INTRODUCTION

The aromatic and medicinal plants with significant industrial interests such as thyme, oregano, sage, and rosemary have also been reported to possess diverse biological properties including antioxidant and antimicrobial activities (Ruiz-Navajas et al., 2012). Thyme is a short shrub of the genus (*Thymus vulgaris* L.) containing 350 species of perennial herbaceous plants. Due to its distinctive aromatic flavouring, it has been used in stuffing, meats, sauces, stews, and poultry – indeed for almost anything from soup to salad (Dobelis, 1986; Lorenzo et al., 2018). Mostly, extract of *Thymus vulgaris* is used for the treatment of gastritis, diarrhoea, bronchial problems, and whooping cough (Rehan et al., 2018). Thyme leaves have commonly been used in foods mainly for their flavour, aromas and preservation, herbal tea for a long time in Turkey (Sagdic, 2003). Furthermore the components of thyme include several bioactive compounds such as phenolic acids (rosmarinic acid, caffeic acid, *p*-hydroxybenzoic acid, etc), flavonoids (luteolin, apigenin, thymonin, etc), biphenyl compounds, triterpenes (ursolic acid, oleanolic acid), and essential oils (thymol, carvacrol) (Lorenzo et al., 2018).

Bioaccessibility is associated with the amount of bioactive constituents present in the gut as a result of their liberation from the food matrix due to the conditions related to gastric and intestinal medium. It is an important aspect that determines the beneficial properties of the bioactive compounds and hence, investigation of their metabolic fate could be crucial to develop novel and more efficient functional foods.

It has been previously reported that the processing of foods, such as drying, canning, high-pressure processing, or fermentation, would influence the initial bioactive composition, antioxidant potential, structure, and thus bioaccessibility of the existing phytochemicals in the raw food (Kamiloglu and Capanoglu, 2013; Yeo and Ewe, 2015; Barba et al., 2017; Escrivá et al., 2021). The phytochemicals that exist naturally in plant foods are mostly in bound form, which is less bioavailable than the free form, and many processing methods could therefore have potential facilitating the release of bound compounds. Ucar and Karadag (2019) determined that drying process would cause the disruption of the cellular structure and *in vitro* bioaccessibility of individual phenolics would be different in dried and fresh samples.

Natural fermentation has been long used in human history to improve the nutritive value of plant foods. During fermentation, with the action of the enzymes produced by the starter microorganisms, the structural breakdown of plant cell walls, the release or synthesis of various antioxidant compounds that may act as free radical terminators, metal chelators, singlet oxygen quenchers, or hydrogen donors to radicals could occur (Hur et al., 2014; Son et al., 2020). Wen et al. (2013) reported that fermentation enhanced the release of functional ingredients from the extracts. Another study reported that the fermentation by *Lactobacillus fermentum* (0.1% inoculum) increased the total flavonoids in red ginseng extract by approximately 39% compared to unfermented red ginseng (Kim et al., 2011).

Thyme has been conventionally consumed in fresh, dried, and fermented forms, and we presumed that the processing, mainly fermentation and drying, would have an important effect on the bioaccessibility of phenolics and their antioxidant activities. Therefore, in this study, the extracts of fresh, dried, and fermented thyme leaves were assessed in terms of total phenolic content, antioxidant capacity, and the change of measured properties after subjecting them to *in vitro* simulated gastrointestinal digestion conditions.

2. MATERIALS AND METHODS

2.1. Chemicals

Folin–Ciocalteu's phenol reagent, sodium carbonate, sodium nitrite, aluminium trichloride, copper(II) chloride, ammonium acetate, sodium bicarbonate, sodium hydroxide, ethanol, hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany), and gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), catechin hydrate standard, neocuproine, pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545), and bile (B8631) were obtained from Sigma- Aldrich Ltd (Steinheim, Germany).

2.2. Preparation of raw material

Fresh *Thymus vulgaris* was obtained from the local vegetable market in Istanbul, Turkey. The moisture content of the fresh thyme was 75 ± 2.05%. The dried samples were prepared at 45 °C for 5 h in a hot-air drier until the final moisture content of 11.16 ± 0.34% was achieved. Fresh thyme leaves were fermented by immersing 150 g of leaves into 90 mL (60:30, v/v) of brine with 6% of salt and grape vinegar (5% acetic acid) in a sterile glass jar. The jars were stored at 20 ± 2 °C for their fermentation for 18 days (Fig. 1).

2.3. Extract preparation

The extracts of the fresh (FT), dried (DT), and fermented-pickled (PT) thyme leaves were prepared as follows: 10 g of each sample was extracted with 100 mL 80% ethanol (1:10, w/v) for 2 h on a magnetic stirrer at 25 °C, and then centrifuged (2,500×*g*, 10 min). The supernatant was collected and the residue was re-extracted twice. The collected supernatants were pooled, the solvent was removed in a rotary evaporator (R-215, Büchi®, Flawil, Switzerland), and the dried residue was used for the assays.

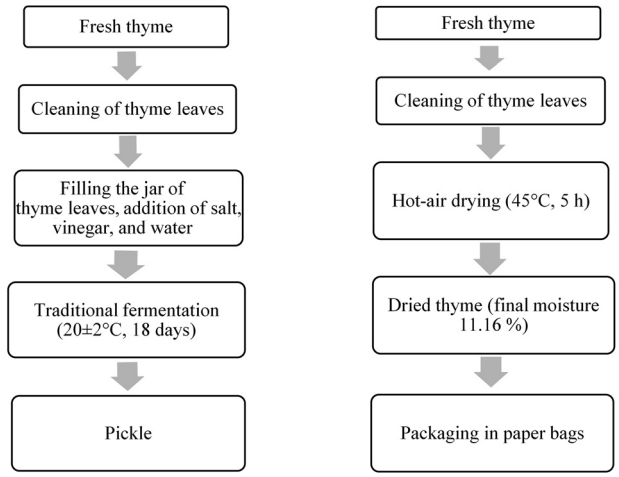

Fig. 1. Flow diagrams of traditional fermentation (left) and hot-air drying (right) of thyme leaves

2.4. Determination of total phenolic and total flavonoid content

Total phenolic content (TPC) of the thyme leaf extracts was determined with the Folin–Ciocalteu (FC) reagent according to the method described by Singleton and Rossi (1965). Gallic acid was chosen as a reference standard. The absorbance was measured at 760 nm using a Shimadzu 150 UV-1800 spectrophotometer (Kyoto, Japan). The results were presented as mg gallic acid equivalent (GAE) per g dry weight (DW). The linear range of the standard curve was from 0.01 to 0.12 mg mL^{-1} ($r^2 = 0.993$). Total flavonoid content (TFC) was determined by the aluminium trichloride ($AlCl_3$) method (Zhishen et al., 1999). The absorbance was measured at 510 nm using a spectrophotometer (Shimadzu 150 UV-1800 spectrophotometer, Japan). The results were given as mg catechin equivalent (CE) per g DW with a linear range of 0.01–0.35 mg mL^{-1} ($r^2 = 0.996$).

2.5. Antioxidant capacity assays

The scavenging activity of the thyme leaf extracts against DPPH radical was detected as absorbance by a spectrophotometer at 517 nm (Singh et al., 2002). The results were calculated as μmol Trolox equivalent (TE) per g DW. The linear range of the standard curve was from 200 to 1998 μM ($r^2 = 0.996$). According to the protocol described by Apak et al. (2004), the cupric ion reducing antioxidant capacity (CUPRAC) of the thyme leaf extracts was determined. The absorbance was measured at 450 nm using a Shimadzu UV-1800 spectrophotometer. The results were given as μmol TE per g DW with a linear range of 200–2000 μM ($r^2 = 0.990$).

2.6. Simulated *in vitro* gastrointestinal (GI) digestion assay

The *in vitro* gastrointestinal digestion model of McDougall et al. (2005) was applied for FT, DT, and PT extracts. Change in antioxidant capacity and release of phenolics of extracts were examined at a gastric and an intestinal stage of digestion. Briefly, 2.5 mL of extracts were mixed with 20 mL of distilled water, and the pH was adjusted to 2 with 5 M HCl, 315 units/mL pepsin was added, and then it was incubated at 37 °C in a heated water bath for 2 h with shaking at 100 r.p.m. After 2 h, 2 mL aliquots of the post-gastric (PG) digestion were collected. 4.5 mL of 4 mg mL^{-1} pancreatin (2 units/mL) and 25 mg mL^{-1} bile salt mixtures were added to the remainder in the 250 mL glass beaker. Segments of dialysis bags (MWCO 12,000 Da) were cut and filled with sufficient sodium $NaHCO_3$ (0.5 M) to neutralise the samples' titratable acidity (pH 7). Samples were incubated in a shaking water bath (100 r.p.m.) at 37 °C for another 2 h to complete the intestinal phase of the *in vitro* digestion process. After the intestinal phase, the content of the dialysis membrane was taken as the "serum-available" and "bioavailable" content (IN), while the medium outside the membrane was referred to as "colon-available" content (OUT). The blank was also prepared with identical chemicals but without samples and underwent the same procedures. Then the sample taken at each digestion step was centrifuged at 2,700×*g* for 10 min and stored at –20 °C for the analysis. The effect of *in vitro* digestion on the phenolics and antioxidant capacity of thyme samples was evaluated by the Bioaccessibility index (BI%) (Equation 1) and Recovery percentage (R%) (Equation 2):

$$\text{BI\%} = \frac{(\text{IN} + \text{OUT})}{\text{Undigested}} \times 100 \quad (1)$$

$$R\% = \frac{IN}{Undigested} \times 100 \quad (2)$$

2.7. Statistical analysis

Statistical analysis was performed using SPSS Statistics (IBM SPSS 17.0, USA). All data were presented as a mean of three measurements, *i.e.*± standard deviation. The differences among the FT, DT, and PT were evaluated by analysis of variance (ANOVA) combined with the Duncan comparison test at $P < 0.05$ significance level.

3. RESULTS AND DISCUSSIONS

3.1. Total phenolic, total flavonoid contents, and antioxidant capacity of thyme extracts

The total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, and CUPRAC values of fresh, dried, and fermented-pickled thyme extracts are summarised in Table 1. The results indicated that TPC, TFC, and antioxidant capacities varied

Table 1. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities of thyme extracts submitted to *in vitro* digestion

Analysis	Initial	Gastric	Intestinal	
			IN	OUT
TPC (mg GAE/g DW)				
FT	46.72 ± 1.34^{cA}	6.25 ± 0.03^{bB}	5.95 ± 0.06^{bC}	8.44 ± 0.09^{bB}
DT	52.34 ± 0.60^{bA}	3.46 ± 0.05^{cB}	2.32 ± 0.02^{cC}	3.24 ± 0.11^{cB}
PT	71.09 ± 0.53^{aA}	10.45 ± 0.07^{aC}	19.66 ± 0.26^{aB}	10.90 ± 0.10^{aC}
TFC (mg CE/g DW)				
FT	14.85 ± 0.17^{bA}	1.01 ± 0.04^{bB}	0.37 ± 0.10^{bC}	0.83 ± 0.10^{bB}
DT	10.29 ± 0.48^{cA}	0.17 ± 0.03^{cC}	0.09 ± 0.00^{cC}	0.74 ± 0.04^{bB}
PT	30.89 ± 0.71^{aA}	3.88 ± 0.11^{aC}	6.14 ± 0.21^{aB}	3.71 ± 0.07^{aC}
DPPH (μmol TE/g DW)				
FT	40.16 ± 0.40^{cA}	11.76 ± 1.11^{bB}	4.56 ± 0.40^{cC}	2.56 ± 0.53^{cD}
DT	59.54 ± 0.71^{bA}	10.15 ± 0.36^{bB}	8.73 ± 0.22^{bC}	6.22 ± 0.14^{bD}
PT	93.58 ± 5.86^{aA}	47.57 ± 1.21^{aB}	28.87 ± 0.22^{aC}	23.65 ± 0.22^{aC}
CUPRAC (μmol TE/g DW)				
FT	75.80 ± 1.22^{cA}	16.93 ± 0.20^{bC}	11.20 ± 0.25^{bD}	24.63 ± 0.95^{bB}
DT	137.59 ± 0.28^{bA}	9.16 ± 0.17^{cB}	6.67 ± 0.13^{cD}	7.27 ± 0.07^{cC}
PT	179.66 ± 7.50^{aA}	34.05 ± 0.11^{aB}	26.55 ± 2.61^{aB}	30.11 ± 0.23^{aB}

Data are expressed as mean ± S.D. of triplicate measurements. Different letters (a-c) in the same column are significantly different ($P < 0.05$) among thyme samples. Different capital letters (A–D) in the same row are different ($P < 0.05$) for the same sample at each digestion phase. FT: fresh thyme; D: dried thyme; PT: fermented-pickled thyme; TPC: total phenolic contents; TFC: total flavonoid contents.

significantly among the three thyme extracts (P <0.05). The TPC of FT, DT, and PT extracts were found as 46.72 ± 1.34, 52.34 ± 0.60, and 71.09 ± 0.53 mg GAE/g DW, respectively. Our results were in agreement with the study of Nikolic et al. (2019), who reported TPC of fresh *Thymus vulgaris* as 13.36 mg GAE/g fresh weight (FW), and also Nateqi and Mirghazanfari (2018), who determined TPC of the dried *Thymus vulgaris* hydro-ethanolic extract as 68.34 mg GAE/g DW. TPC result (52.34 ± 0.60 mg GAE/g DW) determined in our study for dried thyme extract was higher than those determined by Georgieva and Mihaylova (2015) (11.16 ± 2.76 mg GAE/g DW) and Rehan et al. (2018) (1.2 mg GAE/g DW). The TFC of fresh, dried, and fermented thyme extracts were found as 14.85 ± 0.17, 10.29 ± 0.48, and 30.89 ± 0.71 mg CE/g DW, respectively. Other reported TFC data for dried *Thymus vulgaris* were between 0.8 and 17.64 mg CE/g DW (Abdul Qadir et al., 2017; Rehan et al., 2018).

The DPPH radical scavenging activity of PT extract (93.58 ± 5.86 µmol TE/g DW) was significantly higher than those of dried (59.54 ± 0.71 µmol TE/g DW) and fresh (40.16 ± 0.40 µmol TE/g DW) thyme extracts. Likewise, the same trend was observed with the CUPRAC value, in which fermented thyme exhibited significantly higher antioxidant capacity (179.66 ± 7.50 µmol TE/g DW). Higher antioxidant capacity observed in fermented thyme leaves positively correlated with the increase in amount of polyphenols, which was also previously reported for medicinal plants (Yashin et al., 2017; Tungmunnithum et al., 2018).

In our study, the CUPRAC values of both extracts were higher than the values obtained by the DPPH method. Cu^{2+} ion takes part in the formation of free radicals; the reduction of cupric ion indicates another mechanism than that of the DPPH method reflecting the antioxidant potential. This observation may be related to the fact that while CUPRAC assay can measure both the hydrophilic and lipophilic antioxidant capacities of the extracts since the reagent is soluble in both aqueous and organic solvents, DPPH uses a radical dissolved only in the organic solvent and therefore might better represents the lipophilic antioxidants (Capanoglu et al., 2018). Nateqi and Mirghazanfari (2018) reported the IC50 values of the DPPH test for dried *Thymus vulgaris* (hydro-ethanolic) extract as 27.68 mg ascorbic acid/g DW and Rehan et al. (2018) reported that the DPPH radical inhibition activity was 49.86% for 25 µg extract/mL. Nikolic et al. (2019) determined DPPH and CUPRAC values of fresh thyme samples as 4.16 mg TE/g FW and 1.48 mg TE/g FW, respectively (Nikolic et al., 2019). Rosmarinic acid was reported to be the major phenolic compound in thyme leaves (6.3–20.9 mg g^{-1}), and it was reported that its contribution to the DPPH activity of the 14 thyme ethanolic extracts ranged from 22 to 55% (Chizzola et al., 2008).

Khouya et al. (2015) determined that three Moroccan thymus varieties possess considerable antioxidant activities and were rich in total polyphenol and flavonoid compounds. Rosmarinic acid was the major phenolic acid, and the extracts were also rich in quercetin and luteolin-7-glucoside.

Köksal et al. (2017) stated that water and ethanol extracts of air-dried thyme leaves were rich in gallic acid, ferulic acid, caffeic acid, ellagic acid, *p*-coumaric acid, and pyrogallol, and those extracts were effective DPPH radical scavengers and presented IC50 values comparable to the standard antioxidants such as Trolox, BHA, BHT, and α-tocopherol. The reducing power of cupric ion ethanolic thyme extracts was reported higher than Trolox and α-tocopherol, but lower than BHT.

TPC, TFC, and antioxidant capacity values of fermented-pickled thyme samples (PT) were significantly higher than those of fresh and dried samples (Table 1). This situation could be

explained with the effects of fermentation on the liberation of bound phenolics that were involved in the formation of molecular bonds among cellulose, hemicellulose, and lignin in cell walls. The enzymes produced by microorganisms, such as β-glucosidase, may catalyse the release of those phenolics, which are usually conjugated with sugar and glycosides via hydroxyl groups. Similarly, the release of ferulic acid, caffeic acid, or *p*-coumaric acid from conjugated phenolic acids by the action of feruloyl esterase enzymes, and the liberation of gallic and ellagic acids, flavonoids, and flavon-3-ols through the activity of esterases produced by the microbial action during fermentation were also previously reported (Septembre-Malaterre et al., 2017). It has also been reported that fermentation of thyme with lactic acid bacteria may induce the release of ferulic acid from the plant cell wall and increase its solubility (Hussain et al., 2016).

Additionally, the structural changes occurring during both drying and fermentation may increase the extractability of bioactive compounds. Guldiken et al. (2016) determined the total phenolic and total flavonoid contents and antioxidant activities of fresh, dried, and fermented red beetroot, and reported that on a dry basis the pickled red beet showed a higher amount of TPC compared to the fresh sample, although the pickled samples were cooked at 200 °C for 20 min previously.

3.2. Simulated *in vitro* gastrointestinal digestion assay

The change of TPC, TFC, and antioxidant capacity values of FT, DT, and PT during each step of *in vitro* gastrointestinal digestion (gastric and intestinal phases; the material entered the serum (IN) and the material remained in the GI tract (OUT) after intestinal digestion) are given in Table 1. After gastric digestion (PG), compared to the initial values, a significantly lower ($P < 0.05$) amount of TPC (13.4%, 6.6%, and 14.7%) and TFC (6.8%, 1.6%, and 12.5%) were recovered from FT, DT, and PT extract, respectively. A similar trend was observed for antioxidant capacity, values obtained after PG were significantly lower (6.6–50.8% recovery) ($P < 0.05$) compared to the initial values. It may be a result of the low solubility of the extracts in the gastric medium. At the intestinal stage, if we consider the sum of IN and OUT fractions, it can be said that the recovery % compared to the initial becomes higher, due to possibly higher solubility of phenolics in the intestinal stage as a result of pH and the action of bile salt. Interestingly, at the intestinal phase, PT showed a different trend than other samples, In the IN fraction, TPC and TFC values were significantly higher than those of OUT fraction, whereas in other samples the trend was the opposite. It might show that the products of FT extracts could possibly have less polymerised structure and lower molecular weight and be able to pass the membrane. It was reported that the factors affecting the recovery of phenolics were related to their chemical structure, solubility, molecular weight, and the presence of the glycosidic group attached to them (Tayiroğlu and Incedayı, 2020). Although the differences of antioxidant capacity values between IN and OUT fractions provided by PT were not significant, for other samples, the DPPH values obtained for OUT fractions were lower than those of IN fractions, whereas in terms of the CUPRAC values, OUT fractions provided higher values. It might indicate that the different individual phenolics and their possible structure obtained after being submitted to digestion provided different antioxidant capacity values, therefore total TPC and TFC values may only give limited information, but still can be used for screening purposes.

The recovery % is given as the ratio of the values determined for the IN fraction to the values determined for initial (hydro-ethanolic extract) values and then multiplied by 100 (Fig. 2).

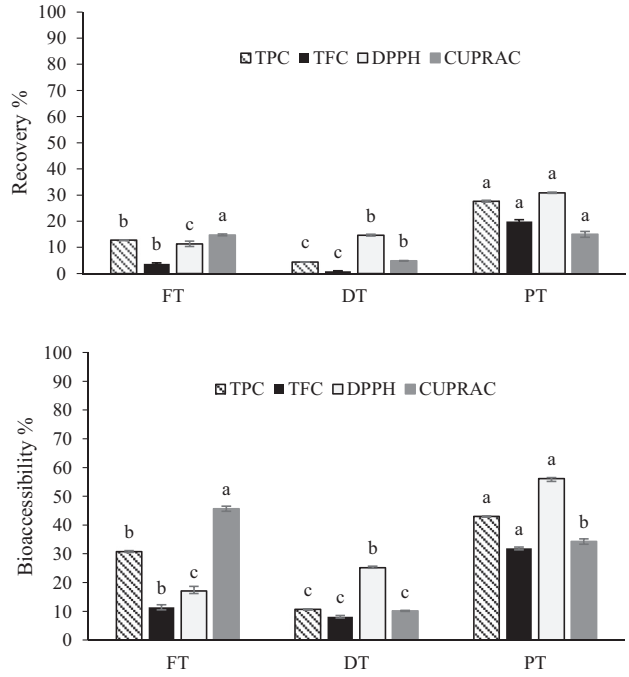

Fig. 2. Recovery % and the bioaccessibility of thyme extracts. Data represent average values ± standard deviation. Different letters above the bars represent statistically significant differences ($P < 0.05$). FT: fresh thyme; DT: dried thyme; PT: fermented-pickled thyme; TPC: total phenolic contents; TFC: total flavonoid contents; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; CUPRAC: Copper reducing antioxidant capacity; Bioaccessibility %: the values obtained in the (IN+OUT) fraction/the values obtained in the initial sample; Recovery %: the values obtained in the IN fraction/the values obtained in the initial sample

According to Fig. 2, the highest recovery values of TPC and TFC were found in PT extract (27.6 and 19.9%, respectively), while the lowest recovery values were found in DT extract (4.4% and 1.0%, respectively). When the bioaccessibility indices (BI), the ratio of the intestinal stage to the initial undigested sample, were evaluated, a similar trend was observed, and in PT extracts the BI were determined as 43% and 32% for TPC and TFC, respectively, and the lowest values were determined in DT sample (10.6% and 8.1%, respectively). It might be the case that heating of phenolics during drying of thyme samples may produce some polymerised substances with lower solubility and absorption. Rubió et al. (2014) studied the effect of the co-occurring bioactive compounds of dried thyme on bioaccessibility through an *in vitro* human digestion model and, similarly to our results, determined the bioaccessibility of TFC as 9.5%. As for the evaluation of antioxidant capacities of the samples by DPPH, both the recovery % and the BI % obtained were highest in PT (31 and 56%, respectively) and the lowest in FT (11 and 17%, respectively). However, when the CUPRAC assay was applied, the recovery % for FT and PT were similar (15%), and the BI % was higher for FT. It should be born in mind that those indices are not the direct values representing the antioxidant activities of the samples in the intestinal

stage, rather their ratio to the initial values. For example, the CUPRAC value provided by PT intestinal digestion (56.6 μmol TE/g DW) was significantly higher than of FT sample (35.83 μmol TE/g DW). Its bioaccessibility index was lower because the initial amount in the undigested sample was more than twice that of the FT sample. It has been also stated in the literature that the antioxidant capacity assay conditions such as pH, organic solvent dependency, and the presence of artefacts in the medium, their mechanism of action, the changed reactivity of the compounds after digestion would influence the results that should be interpreted carefully. Therefore, most accurate evaluation of the antioxidant capacity of foods has been suggested using a variety of assays with different mechanisms (Kamiloglu et al., 2015).

4. CONCLUSIONS

Overall, the results of our study indicated that fermentation applied to produce fermented-pickled thyme leaves both increased the TPC, TFC, and antioxidant activities of undigested samples and the *in vitro* bioaccessibility of the samples through the simulated digestion model. For dried thyme leaves, although the undigested sample generally showed higher TPC and antioxidant capacity values than the fresh sample, possibly the drying conditions promoted the degradation of cellulosic material and changed the matrix, therefore, increased the extractability. However, in the digestive fluids, the measured values were low for dried samples. Possibly the polymerised structures formed during drying have lower solubility and antioxidant capacity values. This study revealed that both drying and fermentation had a considerable effect on the bioactive properties of undigested thyme samples, and the fate of the samples subjected to *in vitro* simulated gastrointestinal digestion also differed depending on the processing applied, e.g, being fresh, dried, or fermented. In the light of these data, future studies should focus on improving production techniques for medicinal plants.

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