Trends Phytochem. Res. 2(1) 2018 13-20

Trends in Phytochemical Research (TPR)

Journal Homepage: http://tpr.iau-shahrood.ac.ir

Original Research Article

Effect of extraction methods on phenolics, flavonoids and antioxidant activity of *Thymus vulgaris* and *Origanum vulgare* extracts

Suzara R.C. Sena, Theresa R.F. Dantas and Camila G. Pereira ✉

Laboratory of Separation Process in Foods, Dep Chemical Engineering, Federal University of Rio Grande do Norte- UFRN, CEP 59072-970 Natal, Brazil

ABSTRACT

The aim of this study was to quantify the amount of phenolic compounds and flavonoids in the extracts from *Thymus vulgaris* and *Origanum vulgare* L. as well as to evaluate the antioxidant activity of these extracts. The extracts were obtained by employing three different techniques: low pressure solvent extraction, Soxhlet, and ultrasound methods. Essential oils were also obtained by hydrodistillation to provide a comparison between the species. The effects of phenolic and flavonoid concentrations on the percentage of inhibition of free radical activity were also analyzed. The highest amounts of phenolic compounds were obtained with Soxhlet and Low Pressure Solvent Extraction (LPSE) for thyme and oregano, respectively. The highest flavonoid levels were obtained by the Soxhlet extracts for both species. All the extracts showed strong antioxidant activities.

ARTICLE HISTORY

Received: 26 August 2017
Revised: 10 January 2018
Accepted: 28 January 2018
ePublished: 07 March 2018

KEYWORDS

Thymus vulgaris
Origanum vulgare L.
Phenolic compounds
Flavonoids
Antioxidant activity
Separation processes

1. Introduction

The presence of bioactive compounds in foods is a possibility to improve public health through the diet. Research has shown that many types of food contain compounds with antioxidant action, as is the case of condiments. These plants are very appreciated by their culinary applications and their medicinal properties. The antioxidant effect of 32 different spices was published in 1952 by Chipault and colleagues (Melo et al., 2003). Since then, other beneficial health effects have been shown for different spices.

Spices are defined as aromatic plants that may or may not be "spicy" but are used to impart flavor and fragrance to food (Bedin et al., 1999; Mohammadhosseini, 2017; Mohammadhosseini et al., 2017). Among several species of spice plants, two are commonly used in the human diet: oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*).

The *Origanum* genus is a perennial herb native to Europe and Siberia that includes 38 species (Aligians et al., 2001; Fernandes et al., 2017). Due to its variety of flavors, chemical characteristics and biological effects, different species and biotypes of *Origanum* are extensively used in the pharmaceutical, cosmetic and food industries (Sivropoulou et al., 1996; Novack et al., 2000; Aligians et al., 2001; Ozcan et al., 2017). For instance, the oregano effects range from digestive and antiseptic (Çabuk et al., 2003) to bactericidal (Friedman et al., 2002; Burt and Reinders, 2003; Rhayour et al., 2003; Özkalp et al., 2010; Ozcan and Ozkan, 2015), analgesic (Seoudi et al., 2009), antifungal (Soliman and Badea, 2002; Pina-Vaz et al., 2004), antioxidant (Vekiari et al., 1993; Earchou et al., 2002; Faiku et al., 2017), and expectorant (Fernandez et al., 2009). These effects may be attributable to groups of compounds found in the spice such as essential oils, phenolic acids, flavonoids and tannins (Sagdiç, 2003).

Another important spice is thyme (*Thymus vulgaris* L.). This spice is widely used in folk medicine (Rezaei and Mohammadhosseini, 2014; Hashemi-Moghaddam et al., 2015; Mohammadhosseini, 2016). The oil of thyme has antiseptic, bactericidal, antifungal, anti-hemolytic, expectorant, carminative and antispasmodic actions

✉ Corresponding author: Camila Pereira
Tel: +55 084 3215-3773; Fax: +55 084 3215-3770
E-mail address: camila@eq.ufrn.br

(Vieira de Melo et al., 2000; Simões et al., 2004; Kasrati et al., 2017; Mazandarani and Ghafourian, 2017). The hydroalcoholic extract from thyme was found to inhibit the growth of *Candida albicans* (Nascimento et al., 2000; Bonjar, 2004), *Aspergillus flavus* (Bonjar, 2004), *Pseudomonas aeruginosa* and *Proteus* spp. (Nascimento et al., 2000) as well as *Streptococcus mutans* and *Streptococcus sobrinus* (Gebara et al., 1996). Another study indicated that the essential oil of thyme has antifungal activity against several species of *Candida*, including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilhermondii* and *C. parapsilosis* (Pina-Vaz et al., 2004).

Furthermore, studies also have shown the higher capacity of oregano and thyme to inhibit the growth of *Escherichia coli* O157:H7 (Sağdiç et al., 2002).

The extraction of the active compounds in these spices depends on the processing and fractionating methodologies used. Although there are many papers about thyme and oregano, up to now, none of them have made an exhaustive comparison in relation to the extraction technique of the total phenolic and flavonoid compounds, for their potential use in food and pharmaceutical products. Given the important biological characteristics of these plants and the possibility of their use as ingredient in various food and beverages, the optimization of the extraction of specific compounds is desirable and essential to take full advantage of the properties of such compounds. Thus, the objective of this study was to evaluate the phenolic and flavonoid contents in thyme and oregano extracts obtained by different techniques and to determine their antioxidant activities. For the extraction processes evaluated in this work, ethanol was used as solvent because it is considered as a GRAS (Generally Recognized As Safe) solvent by FDA (Food and Drug Administration).

2. Experimental

The entire experimental analytical step (characterization, separation processes, analyses of the extracts, and antioxidant activity evaluation) was performed in triplicate.

2.1. Preparation and characterization of the raw material

Raw thyme (lot 090603), *Thymus vulgaris*, and oregano (lot 092403), *Origanum vulgare* L, from Peninha Alimentos (Natal, Brazil), were prepared and characterized in terms of particle size distribution (PSD), real density of the particles, apparent bed density, bed plus particles porosity and humidity. The characterization of the raw material and pre-processing (selection, partition, comminution, drying, etc), if necessary, are essential steps when evaluating a process so that it actually has an industrial application. The characterization provides important parameters of the raw material before the extraction process is carried out.

Particle size distribution (granulometry) was determined using a mechanical agitator (Bertel, model NOVO 110/220, São Paulo, Brazil) with standard sieves of the Tyler series (10, 24, 32, 48, and 80 mesh). The particle size is the measure of the size of the raw material (whole and broken leaves) used in the experiments of extraction. As the particles have different sizes, this analysis provides an information in relation to the medium size of these particles and how they are distributed in the material used in the experiments.

The real density of the particles was measured by using the method described by Buczek and Geldart (2000). The apparent bed density was calculated using the mass of feed and volume of the extractor cell. The bed plus particles porosity was calculated as the ratio of the apparent bed density and real density. The humidity was determined by Jacobs method (1973).

2.2. Extraction Methods

2.2.1. Low Pressure Solvent Extraction (LPSE)

The raw materials (10 g) were suspended in ethanol (PA, Cromato Produtos Químicos Ltda., São Paulo, Brazil, lot 1188.05/08) using a solid:solvent ratio of 1:10. Each solution was placed in a shaker (Tecnal, TE-422 model, São Paulo, Brazil) for 2 h, and maintained at room temperature (21 ºC) and pressure (1.01 bar). The solutions were then filtered using a gauze and the solvent was evaporated in a vacuum evaporator (Heidolph, Rotavapor Laborota 4000 OB, Schwabach, Germany). The extractions were performed at 40 ºC.

2.2.2. Soxhlet extraction

Approximately, 10 g of the raw material was extracted separately in 100 mL of ethanol for 3 h. The extractions were carried out at the boiling temperature of the solvent. After extraction, the solvent was evaporated using a vacuum evaporator.

2.2.3. Ultrasound extraction

Approximately 1 g of the raw material was extracted in 10 mL of ethanol (PA, Química Fina, lot L081106, Diadema, Brazil) using an ultrasound apparatus-bath (Unique, Metasom 14, São Paulo, Brazil) with control of temperature. The extraction was carried out at 42 ºC for 60 min. After that, the material was filtered through filter paper using a vacuum system. The solvent was then evaporated using a vacuum evaporator.

2.2.4. Hydrodistillation

Approximately 20 g of the raw material was distilled in 200 mL of water in a hydrodistillator (TE-2761, Tecnal, São Paulo, Brazil) for 3 h. The process was carried out at the boiling temperature of the solvent. After the extraction, the volatile oil and water fractions were collected and separated using methyl chlorate (PA, Vetec, lot 080188, Rio de Janeiro, Brazil), according to Moura et al. (2012) and Leitão et al. (2013).

In all separation methods, the global yield was calculated by the ratio between the mass of extract and the initial mass of raw material, in percentage.

2.3. Extract analysis

2.3.1. Thin layer chromatography

The extracts were analyzed by thin layer chromatography (TLC) using different eluents and detection solutions. TLC analyses were performed using 5 mg of extract diluted in 1 mL of ethyl acetate (PA, Vetec, lot 0804680, Rio de Janeiro, Brazil). Samples were applied to silica gels for separation (5×20 cm, Silica gel 60 com UV254, lot 711331, Macherey-Nagel). Essential oils and flavonoids were separated using 80:20 hexane (Vetec, lot 072203/08, Rio de Janeiro, Brazil): ethyl acetate (PA, Vetec, lot 0804680, Rio de Janeiro, Brazil) as the eluent. Essential oils were detected using an anysaldehide solution. Flavonoids were detected using a solution of oxalic acid in ethanol. All plates were heated to 100 ºC and spots were observed with UV 366 nm light. Quercetin hydrate (95+%, Sigma-Aldrich, lot S43521-367, EUA) was used as the standard.

2.3.2. Quantification of total phenolic compounds

Total phenolics were determined using the methodology described by Singleton and Rossi (1965) with modifications. About 1 mL of the extract and 1 mL of the Folin-Ciocalteau reagent were mixed. After 5 min, 1 mL saturated solution of Na_2CO_3 (approximately 35% in methanol- PA ACS; Vetec, lot 0904809, Rio de Janeiro, Brazil), was added and the volume was brought to 25 mL with methanol. The mixture was protected from light and incubated for 90 min. The absorbance was read at 750 nm using a spectrophotometer (Varian 50, UV, visivel, São Paulo, Brazil). Gallic acid (Vetec, lot 0806387, Rio de Janeiro, Brazil) was used as the standard. The standard curve was determined using gallic acid diluted in methanol in the following concentrations 20, 40, 60, 80, 100, 120 mg/L and 1 mL of the Folin-Ciocalteau reagent (Pereira et al., 2008).

2.3.3. Quantification of flavonoids

Initially, the standard curve was determined using quercetin dihydrate (Quercentin hydrate 95%, Sigma-Aldrich, lot S43521-367, EUA) as the standard substance. For that, aliquots of 2-6 mL of an ethanolic solution (PA, Cromoline, Química Fina, lot 20777/09, Diadema, Brazil) with 50 µg/mL quercetin were mixed with 1 mL of 2.5% $AlCl_3$. The final solution was taken to 100 mL with ethanol. After 30 min, the absorbance was read at 425 nm using a spectrophotometer. The quantification of the flavonoids present in the extracts was performed using 2 mL of the 2 mg/mL extract solution (obtained by dissolving 100 mg of dry residue in 50 mL of ethanol) and then analyzed in a spectrophotometer (Vennat et al., 1992; Woisky and Salatino, 1998; Brasil, 2001).

2.4. Antioxidant activity

The antioxidant activities (AAs) of the extracts were determined using the methodology of Brand-Williams et al. (1965) as modified by Herrero et al. (2004). The method consists of the neutralization of free radicals of DPPH (2,2´-diphenyl-1-picrylhydrazyl, lot 07717TH, Sigma-Aldrich, EUA) through a decrease in the absorbance at 516 nm. First, a solution of 0.0214 mg/mL of DPPH in methanol (Vetec, lot 0904809, Rio de Janeiro, Brazil) was prepared. 3.9 mL of the DPPH solution was mixed with 0.1 mL of the extract solution with concentrations varying from 10 mg/mL to 0.5 mg/mL, forming the reaction solution with a total of 4 mL. The reaction time was 4 h at room temperature (21 ºC). The absorbances of the samples were read at 516 nm. The antioxidant activity of each extract was calculated in terms of percentage of inhibition by Eqn. 1:

$$AA\%=100-\{[(Abs_{sample}-Abs_{control})\times 100]/Abs_{DPPH}\} \quad \text{(Eqn.1)}$$

Where Abs_{sample} is the absorbance of the sample, $Abs_{control}$ is the absorbance of the control solution and AbsDPPH is the absorbance of the DPPH.

The IC_{50} value was also calculated for all samples. This value represents concentration of the antioxidant to reduce the DPPH radical by 50%.

3. Results and Discussion

3.1. Characterization of the raw material

The characterization of the raw spices is presented in Table 1. These properties are coherent with the values expected for this kind of products (Pereira, 2011).

Table 1
Characterization of thyme and oregano.

Properties	Thyme	Oregano
Granulometry- in mesh (%)	24 (60.8%), 28 (30.9%) 32 (4.6%), 48 (3.7%)	24 (37.3%), 28 (33.5%) 32 (29.1%), 48 (19.9%)
D_{st} (mm)	0.6598	0.6149
Humidity (%)	10.5 ± 0.2	14 ± 3
Real density (g/cm^3)	0.363 ± 0.002	0.354 ± 0.05
Apparent density (g/cm^3)	0.266	0.160
Porosity (ε)	0.267	0.548

3.2. Comparisons of the extraction procedures

Table 2 presents the total yields of the different extraction processes for thyme and oregano. The Soxhlet method produced the highest global yields for both thyme (9.7 ± 0.2%) and oregano (8.4 ± 0.1%).

Determining the best technique should not only be based on the global yield, but an analysis of the chemical profile is also required. TLC analysis (plates not illustrated in this work) indicated that all essential oils and flavonoids are present in all organic extracts. However, very low visual differences between the extracts were observed. It is also known that hydrodistillation is a technique that recovers only essential oils, thus, the material obtained by this method was not used to evaluate the total phenolic and flavonoid compounds. However, it is possible to observe from Table 2 that thyme provided much more essential oils than oregano.

Table 2

Global yield of extract and essential oil* from thyme and oregano obtained by different separation processes.

Process	Global yield (%)	
	Thyme	Oregano
LPSE	5.3 ± 0.1	6.2 ± 0.6
Soxhlet	9.7 ± 0.2	8.4 ± 0.1
Ultrasound	6.4 ± 0.4	5.6 ± 0.6
Hydrodistillation*	0.9 ± 0.1	0.005

3.3. Determination of total phenolics

The total phenolic contents in the thyme and oregano extracts are shown in Table 3 and are expressed as mg of GAE (gallic acid equivalents) per g of extract.

The Soxhlet extraction of thyme presented the highest amounts of total phenolics (10.98 mg GAE/g extract), and for oregano the extracts obtained by LPSE presented the highest amounts of total phenolics (25.15 mg GAE/g extract). In all cases, the amount of total phenolics from oregano was greater than the amount from the thyme extracts, with exception that the Soxhlet method produced similar yields. The difference in the extract yields is due to the kind of process used. Each process works differently: the LPSE extraction is based only on the solubility of the solute in the solvent; the Soxhlet method makes the temperature an additional factor which generally improves the extraction however it can lead to the extraction of other compounds that may be undesirable or degraded, thermolabile compounds; the ultrasound technique promotes acoustic cavitation inside the solid, causing shear disruption and thinning of cell membranes thus facilitating the solvent penetration into the cells and intensification of the mass transfer (Takeuchi et al., 2008).

Note that there is no formula for defining the best extraction process. For thyme, the higher temperature used in the Soxhlet extraction facilitates the extraction and promotes a higher amount of total phenolics. On the other hand, the temperature used by the Soxhlet extraction might have extracted other compounds or have degraded some phenolic compounds present in oregano. As for the ultrasound technique, we believe that the level of ultrasound applied to the spices was not enough to get a desirable result. This is because the amount of total phenolics extracted by ultrasound was similar to LPSE for both spices which indicates that the level of ultrasound was not enough.

A literature survey indicates that total phenolic and flavonoid content in oregano extract varied from 23.48 to 98.86 mg GAE/G extract, and from 14.45 to 19.53 mg quercetin/g extract, respectively (Jain and Bhagia, 2016). However, these authors used water and DMSO (dimethyl sulfoxide), different from our study that used ethanol as a solvent in the LPSE processes. As it is known, the choice of solvent affects directly on the selectivity and yield of extraction. Besides, the edaphoclimatic conditions and nutrients in the soil also affect plant development and composition of the extracts (Pereira and Meireles, 2007).

From the TLC analysis, it was possible to observe that the amount of phenolic compounds in the extracts were similar across techniques. However, the TLC is only a qualitative analysis and according to Table 3 it is possible to verify that in fact there are differences in the extracts.

It is also important to note that the global yield (Table 2) is only the first step in choosing the best process to be employed. For thyme, the highest total yield and the highest amount of total phenolics were achieved in the Soxhlet extracts. Nonetheless, for oregano the highest amount of total phenolics was achieved by the LPSE method, which actually showed lower global yield. This reinforces the importance of evaluating the chemical profiles of the extracts in order to avoid making an erroneous conclusion.

Table 3

Yields of total flavonoids and phenolics in thyme and oregano extracts obtained by different separation processes.

Processes	Total phenolics (mg GAE/g extract)		Total Flavonoids (mg/g extract)	
	Thyme	Oregano	Thyme	Oregano
LPSE	5.54	25.15	2.31	1.69
Soxhlet	10.98	10.33	3.63	4.38
Ultrasound	4.13	23.62	1.80	2.12

3.4. Flavonoid determination

Table 3 shows the amount of flavonoids determined in each extract. The highest quantity of flavonoids was obtained in the Soxhlet extracts of both, that approximately doubles the yield of the other extracts. Comparing the species, the Soxhlet oregano extract was 20.7% higher (4.38 mg flavonoids/g extract) than the Soxhlet thyme extract (3.63 mg/g). These amounts

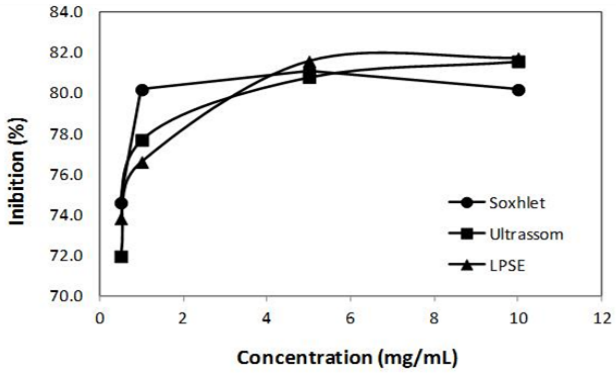

Fig. 1. Inhibition of free radical production by thyme extracts obtained by different techniques. Soxhlet (●); Ultrasound (■); LPSE (▲).

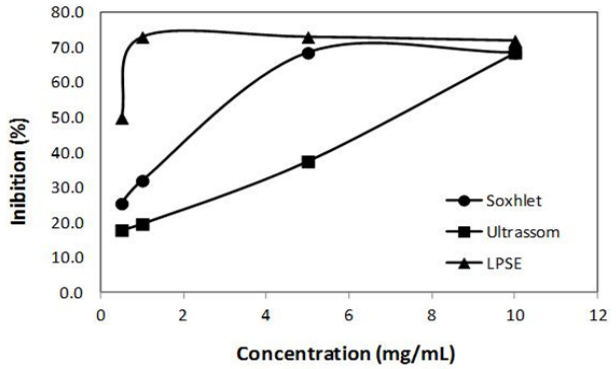

Fig. 2. Inhibition of free radical production by oregano extracts obtained by different techniques. Soxhlet (●); Ultrasound (■); LPSE (▲).

of flavonoids represent around 33.1 and 44.4% of the total phenolic fraction of the thyme and oregano, respectively. These results indicate that other kinds of phenolic compounds in addition to flavonoids were extracted from these spices.

3.5. Antioxidant activity of the extracts

The capacities of the extracts to sequester the DPPH radical are expressed in terms of percentage of inhibition and are presented in Fig. 1 and Fig. 2 as a function of the concentration of the diluted extracts.

According to Fig. 1, the thyme extracts obtained by Soxhlet exhibited more than 80% inhibition with low concentrations (1 mg/mL) and the inhibition was not affected by increasing the concentration. This suggests that its bioactive compounds have effects on the AA independently of their concentrations. This phenomenon has been observed in another study as well (Pereira and Meireles, 2007). The extracts obtained by ultrasound and LPSE, although not as high in the total yield, still had good percentages of inhibition. They showed inhibition around 72-73% at 1 mg/mL, increasing to about 80-81% with 5 mg/mL, and keeping almost constant after that. In this case, the amount of bioactive compounds influenced the AA activity of the extracts. Overall, antioxidant activity was observed in all thyme extracts and was greater than 72% for all extracts at concentrations above 0.5 mg/mL.

The oregano extracts had different behaviors than those observed for thyme (Fig. 2). The highest inhibition was observed for the LPSE extracts, with 49.8% inhibition at 0.5 mg/mL which increased to 72.8% when the concentration was increased to 1 mg/ mL and kept constant after that. The oregano extracts obtained with Soxhlet and ultrasound showed very low inhibitions at 0.5 mg/mL (25.4 and 17.8%, respectively). For the Soxhlet extracts, the percentages of inhibition increased when the concentration was increased from 0.5 to 5 mg/ mL (25.4 to 68.3%) and kept almost constant after 5 mg/mL. The ultrasound extracts were less potent and showed the highest inhibition (68.3%) at 10 mg/mL.

The IC_{50} value for each extract was also determined. For thyme extracts, note in Fig. 1 that all samples presented inhibitions higher that 72% in the minimum concentration evaluated (0.5 mg/mL). The IC_{50} value for all thyme extracts were similar around to 0.34 mg/ mL. For oregano extracts, the IC_{50} values were 0.63, 3.04 and 6.81 mg/mL for LPSE, Soxhlet and ultrasound respectively, confirming that the extract obtained by LPSE provides the greater antioxidant activity compared to the others.

Overall it is possible to observe that the thyme extracts had higher AA than oregano, even though oregano contains higher amounts of flavonoids. This suggests that the amounts of phenolic compounds and flavonoids are not the only factors responsible for AA in these spices. It is known that these compounds have important AA, however, in our study the differences in the amount of phenolic compounds and flavonoids

among the different extracts (for each species) had no influence on the percentage of AA activity. In other words, the antioxidant activity observed in thyme and oregano samples was due to the presence of phenolic compounds as well as other compounds not identified in the extracts. This behavior is common in natural products containing phenolic compounds (Medina et al., 2002; Altunkaya et al., 2009; Pereira and Meireles, 2007; Noguer et al., 2014). Likewise, the AA in these species is associated with the presence of different chemical substances that were acting synergistically, as observed in other species containing such compounds (Altunkaya et al., 2009; Leitão et al., 2013; Noguer et al., 2014).

4. Concluding remarks

In this study, the amounts of phenolic compounds and flavonoids in different extracts from thyme and oregano was determined and the antioxidant activity of these extracts was evaluated. It was observed that global yield of spice extracts was only an initial indicator in the selection of a method to obtain specific compounds. The highest amounts of phenolic compounds were obtained by Soxhlet and LPSE for thyme and oregano, respectively. In contrast, the highest flavonoid amounts were observed in the Soxhlet extracts of both species. Antioxidant activity was observed in all extracts, with values from 71.9 to 81.7% of inhibition for extracts from thyme and from 17.8 to 72.8% of inhibition for extracts from oregano. For thyme, the extract that showed the best AA at low concentrations was that obtained by Soxhlet, and for oregano it was the LPSE extract. It was also observed that the AA activity of the extracts from these species does not depend solely on the concentration of phenolic compounds and flavonoids in the extracts; it was probably due to the presence of other group of compounds acting with synergism.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors are grateful to FAPERN (PPP003/2007) for financial support.

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