

# Antioxidant Properties of Three Aromatic Herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsions

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**Abstract** Lipid oxidation is the major form of deterioration in foods because it decreases food quality and nutritional value, and may have negative health implications. Selected aromatic plant extracts from leaves, flowers and stems of rosemary, thyme and lavender were investigated for their antioxidant activity. The total polyphenol content was determined by the Folin–Ciocalteu assay and the antioxidant capacity was determined by the Trolox equivalent antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl, oxygen radical absorbance capacity and ferric-reducing antioxidant power assays. For all four antioxidant assays, the extracts from thyme flowers, lavender leaves and thyme leaves had the highest antioxidant activity, followed by rosemary stems, rosemary leaves, and lavender stems, and the lavender flowers and thyme stems had the lowest antioxidant activity. The antioxidant activity was correlated with the polyphenol content, although minor deviations were observed. In oil-in-water emulsion, extracts from rosemary leaves and thyme leaves were most effective at retarding oxidation followed by the rosemary stems and thyme flowers. Extracts from thyme flowers and lavender leaves were less effective in the emulsion than predicted by the homogeneous antioxidant assays. This study demonstrated the potential use of plants extract as substitutes for synthetic antioxidants.

**Keywords** Antioxidant · Herbs · Lipid oxidation · Polyphenol · Free radicals · Oil-in-water emulsions

## Introduction

The susceptibility of lipids to oxidation is one of the main causes of deterioration in the quality of food emulsions. This deterioration includes undesirable changes in flavors, textures, shelf life, appearance, and nutritional profiles [1]. In addition, it may cause degradation of nutritional quality, and even affect food safety. Antioxidants are a group of substances that, in low concentrations, inhibit or retard oxidative processes through a mechanism that usually involves oxidation of the antioxidant [2].

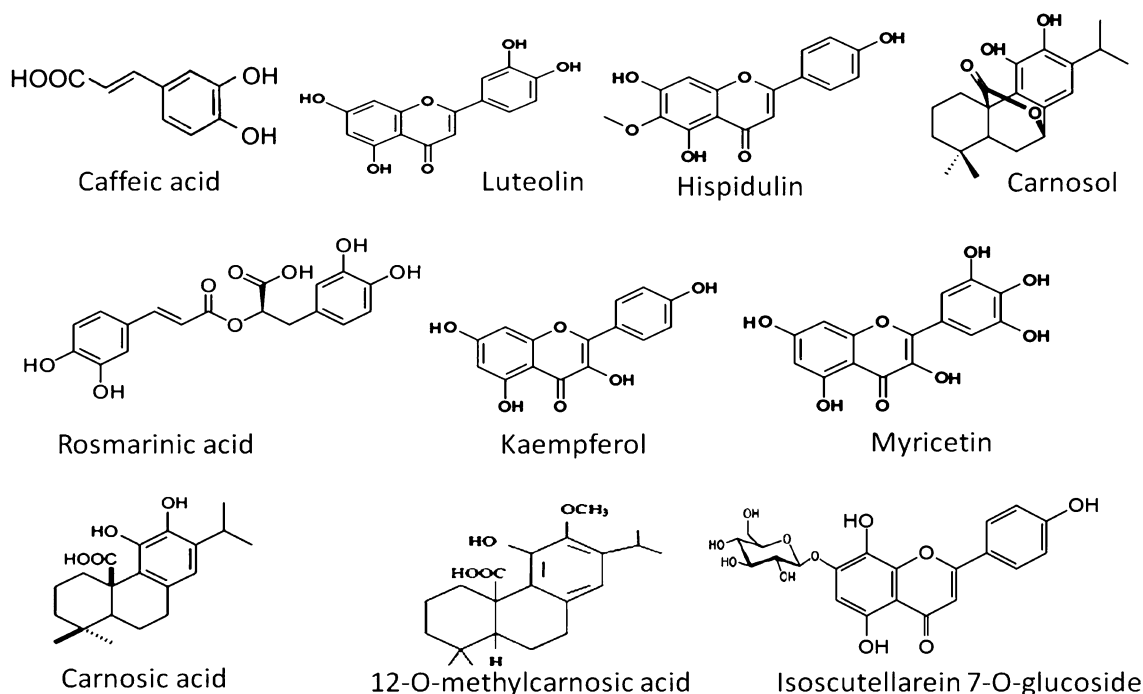
Polar compounds have been reported to impact negatively on the oxidative stability of the oils. Free fatty acids accelerate the oxidation rate of oils and oil-in-water emulsions (O/W) through the ability of the carboxylic acid group to accelerate the decomposition of hydroperoxides and form prooxidative complexes with metals [3].

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are used in many foods to prevent rancidity. However, their effects on health have been questioned. Natural antioxidants are presumed to be safe because they occur in nature and in many cases are derived from plant sources. Natural antioxidants have many advantages: they are accepted by consumers, are considered safe and have less regulatory requirements. The antioxidant properties can be due to many substances including some vitamins, flavonoids, terpenoids, carotenoids and phytoestrogens [4].

The use of herbs and spices to inhibit the development of oxidative reactions in food systems has recently become popular. Rosemary (*Rosmarinus officinalis*), thyme

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**Fig. 1** Structural features of major phenolic compounds in rosemary, thyme and lavender

(*Thymus vulgaris*) and lavender (*Lavandula angustifolia*) have been the center of focus as sources of natural antioxidants, and it has been reported that the antioxidant properties of spices were attributed to their phenolic contents (Fig. 1). The compounds present in these extracts can be classified into three groups: diterpenes, flavonoids and phenolic acids. Compounds such as rosmarinic and carnosic acid are major components [5].

Aromatic herb extracts act as antioxidants to retard oxidation of fats, and their activity can be assessed in O/W as a model system [6]. An emulsion is a dispersion of droplets of one liquid in a second immiscible liquid. It does not form spontaneously and it requires the addition of an emulsifying agent to be stable. The use of emulsions to assess the activity of natural antioxidants is a model for foods such as mayonnaise, where the antioxidant capacity of samples containing additives can be compared with a control emulsion under controlled storage conditions. Lipid oxidation can be monitored in emulsions. In the first phase of lipid oxidation, lipid free radicals react with oxygen to form hydroperoxides [7].

These products usually are further oxidized to form ketones, aldehydes, alcohols and acids that negatively affect the taste, aroma, nutritional value and overall sensory quality of the product, and also lower the pH. Moreover, it is well known that polyphenols and other natural antioxidants significantly enhance the stability of edible oils, because they have the capacity to donate one hydrogen atom to a free radical and reduce propagation of the radical chain reaction [7].

Secondary oxidation of fats can be followed by testing for TBARs (thiobarbituric acid-reactive substances), which has been widely used to determine the degree of oxidative rancidity of a product during storage [8]. Determination of the level of lipid peroxidation in a system can be quantified by measuring the main product formed by the oxidation of polyunsaturated fatty acids, namely malondialdehyde (MDA). This can be detected and quantified when it reacts with thiobarbituric acid. Measurement of thiobarbituric acid reactive substances is a good method to determine the ability of different antioxidants to inhibit lipid peroxidation [8].

The main objective of this study was to determine and compare the antiradical capacity of extracts from three aromatic plants: rosemary (*R. officinalis*), thyme (*T. vulgaris*) and lavender (*L. angustifolia*), with a study of the individual parts (leaf, flower and stem). The antioxidant activity in emulsions was compared in order to demonstrate the ability of extracts from these plants to be used as possible substitutes for synthetic antioxidants in the food industry.

## Materials and Methods

### Plant Material

Aromatic herbs were collected from the region of Barcelona, Catalonia (Spain) in the spring, 2011. The parts (leaf, stem and flower) of the plants were separated. They were then crushed, lyophilized, homogenized and stored (inside

a desiccator) in the dark, at room temperature until use. Plant parts were identified by the nomenclature: RL (rosemary leaf), RR (rosemary stem), TL (thyme leaf), TR (thyme stem), TF (thyme flower), LL (lavender leaf), LR (lavender stem), LF (lavender flower).

### Chemicals

Reagents used were: thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, methanol, hydrogen chloride, aluminum oxide, ferrous chloride, anhydrous sodium carbonate, fluorescein, phosphate buffered saline, ethanol 96 %, ammonium thiocyanate from Panreac. Gallic acid, 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid Trolox, AAPH [2,2'-azobis(2-methylpropionamide) dihydrochloride, 2,4,6-tris (2-pyridyl)-s-triazine], rosmarinic acid, caffeic acid from Sigma-Aldrich.

### Sample Extraction

Plants were weighed (1.5 g) and extracted with 25 mL of solvent. The solvent used was 50 % aqueous ethanol. The solution was stirred continuously for 24 h at 4 °C. After that, all samples were centrifuged. Part of the supernatant was taken for subsequent use to determine the antiradical capacity. The volume of the remaining supernatant was measured and the solution was evaporated, frozen at –80 °C for 24 h and lyophilized for 2 days to provide the sample to be used in the assays. Samples were then weighed and kept protected from light in a desiccator until use.

### Determination of Total Polyphenols

Total polyphenol content was determined by colorimetric spectrophotometry following the method of Folin–Ciocalteu, slightly modified and adapted for microplates. Samples were taken from the extract solutions, diluted 1:25 (V:V), and Folin–Ciocalteu reagent (4 % by volume), sodium carbonate solution 20 % (30.8 % by volume) and Milli-Q water were added. The samples were measured in triplicate. The solution was allowed to react for 1 h in darkness and the absorbance at  $\lambda = 765$  nm was measured.

The gallic acid equivalent concentration was calculated from a standard gallic acid calibration line. The units are expressed as mg gallic acid equivalent per gram of lyophilized extract.

### Determination of the Reducing Capacity by the Ferric-Reducing Antioxidant Power (FRAP) Assay

The determination of reducing capacity was performed with microplates, mixing the FRAP reagent incubated at 37 °C with the samples (in an appropriate dilution to cause the absorbance to fall in the range 0.1–1.0). The FRAP reagent was prepared from 300 mM acetate buffer (279.7 mM acetic acid + 20.3 mM sodium acetate), and 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) and ferric chloride hexahydrate (20 mM). These were mixed in the ratio 10:1:1. The samples were measured in triplicate. The absorbance was measured at  $\lambda = 593$  nm.

### Trolox Equivalent Antioxidant Capacity (TEAC) Assay

A solution (10 mL) containing ABTS radical cation (7 mM) and potassium persulfate (24.24 mM) was prepared. The absorbance of the ABTS radical solution was adjusted by diluting with 10 mM PBS (pH 7.4) incubated at 30 °C. The dilution was adjusted to allow an absorbance of  $0.72 \pm 0.01$ .

The assay was performed in a microplate by mixing the solution containing the radical with the sample extract in appropriate dilutions to allow the rate of fall in fluorescence to lie in the range of the standards. The absorbance was measured in a spectrophotometer at 734 nm for 20 min. The reading after 5 min was taken as the final result, after which there was no decrease in the reading. The samples were measured in triplicate.

From the absorbance data, we determined the percent inhibition of the blank and each sample according to the following expression:

$$\% \text{ inhibition of the blank} = \frac{t_0 - t_5}{t_0} \times 100$$

$$\% \text{ inhibition of the sample} = \frac{t_0 - t_5}{t_0} \times 100 - \% \text{ inhibition of the blank}$$

$t_0$  = the absorbance value at the initial time,  $t_5$  = the absorbance value at 5 min.

From a calibration curve made with the Trolox standard, representing the percentage of inhibition (%) versus the concentration of Trolox (mol/L), the antioxidant capacity of the sample expressed as Trolox equivalents was determined.

### Oxygen Radical Absorbance Capacity (ORAC) Assay

Extract samples were diluted 1:100. They were placed in a microplate and fluorescein ( $8.03 \times 10^{-7}$  M) was added,

with the final proportions in the well of 1/5 (sample) and 3/5 (fluorescein). An initial reading was made with a spectrometer (Fluostar OMEGA, Perkin-Elmer, Paris, France), with the sample prepared at an incubation temperature of 37.0 °C. After this, AAPH (0.3 M) (proportion in well 1/5) was added, and measurement was continued for 2 h. The decrease of fluorescence over time was quantified as area under the curve (AUC). The samples were measured in triplicate.

A calibration curve was prepared using Trolox at different concentrations, with Trolox solution added to give a final concentration in the range between 2 mM and 59 μM, in the well. This curve was specific for the assay of the sample. The ORAC value was calculated as follows:

$$\text{AUC} = \left( \frac{0.5 + \sum_{i=1}^{N_c} f_i}{f_i} \right) \times t_c$$

Decrease<sub>fluorescence</sub> = AUC – AUC<sub>BI</sub>, AUC = area under the curve of the sample in the well, AUC<sub>BI</sub> = area under the curve of the blank,  $f_i$  = fluorescence units ( $f_1$ , is the value of the first reading),  $N_c$  = number of cycles,  $t_c$ : time of each cycle, in this case  $t_c = 2$  (2 min)

From the calibration curve the Trolox concentrations corresponding to the samples under study were determined. The results are expressed as micromoles of Trolox equivalents/g of lyophilized extract

#### DPPH Assay

The DPPH assay determined the ability of extracts to scavenge the DPPH radical. A solution of DPPH (5.07 mM) in pure methanol was prepared. Appropriate dilutions were made for the study of the samples (1:50) to allow the fall in DPPH concentration to be in the range 10–90 %. Then the solution of DPPH and samples (in a concentration of 10 % v/v of sample and 90 % v/v of the radical) were added to the well of a microplate. Absorbance was measured at  $\lambda = 517$  nm, every 15 min for 75 min. The samples were measured in triplicate.

#### Removal of Tocopherols from Sunflower Oil

Alumina was placed in an oven at 200 °C for 24 h, and then it was removed and allowed to cool in a desiccator until it reached room temperature. Sunflower oil was passed twice through the alumina in a column. It was stored at –80 °C until use.

#### Preparation of Emulsions

An oil-in-water emulsion was prepared by dissolving Tween-20 (1 %) in Milli Q water and adding oil (10 %).

The lyophilized extracts were redissolved in 1 mL of ethanol 50 % v/v, and samples were added at a concentration of 100 ppm. A negative control was prepared without the sample and the positive control was prepared from Trolox (250 ppm) dissolved in 1 mL of ethanol. To form the emulsion, the oil was added drop by drop to the solution of Tween 20 in water, which was kept cold, and sonication was continued for 10 min.

The emulsion for each sample was prepared in quadruplicate, obtaining a total of 40 samples. Ethanol extracts of plants and the positive control (Trolox) were solubilized in ethanol. Emulsions were incubated at a temperature of  $33 \pm 1$  °C in an oven, in the absence of light and with constant elliptical movement.

#### Determination of Peroxide Value (PV)

The primary oxidation products were measured using the PV according to the thiocyanate method of the Association of Official Analytical Chemists (AOAC) [9].

Ferrous chloride solution was prepared in hydrochloric acid (1 M) with the addition of iron chloride (II) (2 mM, final concentration). Ammonium thiocyanate solution was prepared in water (2 mM, final concentration).

The assay was performed with a drop of emulsion in the range from 0.007 to 0.01 g, diluted with ethanol. From this solution the required amount of sample varying according to the degree of oxidation was taken in a cuvette and ethanol (96 %) was added. Ferrous chloride and ammonium thiocyanate solutions were added at a concentration of 1.875 % v/v each. The absorbance was measured spectrophotometrically at  $\lambda = 500$  nm.

$$\text{PV} = \frac{\left( \frac{\text{Abs} \cdot \text{dil}}{m} \right) + b}{a}$$

$a$  = represents the slope of the calibration curve, Abs = absorbance,  $b$  = the intercept of the calibration curve, dil = the dilution of each emulsion,  $m$  = weight of the emulsion droplet.

#### Determination of Secondary Oxidation by TBARs

The TBARs reagent was prepared (15 % trichloroacetic acid, 0.375 % thiobarbituric acid and hydrochloric acid 2.1 %). An amount of each emulsion was taken and the TBARs reagent was added in the ratio 1:5. Immediately the samples were added in an ultrasonic bath (Prolabo brand equipment) and immersed in a water bath preheated to 95 °C. Samples were centrifuged and the absorbance of the supernatant was measured at  $\lambda = 531$  nm. The results are expressed as mg MDA/kg of emulsion.

**Table 1** Content of total polyphenols, and antioxidant activity assessed by the ORAC, TEAC, FRAP and DPPH assay for the studied extracts (mean  $\pm$  SD)

|    | Folin–Ciocalteu<br>(mg gallic acid/g<br>lyophilized extract) | TEAC<br>(mM Trolox/g<br>lyophilized extract) | ORAC<br>(mM Trolox/g<br>lyophilized extract) | FRAP<br>(mM Trolox/g<br>lyophilized extract) | DPPH<br>(mM Trolox/g<br>lyophilized extract) |
|----|--|--|--|--|--|
| RL | 219 <sup>e</sup> $\pm$ 6.1                                   | 0.8 <sup>b</sup> $\pm$ 0.04                  | 2.9 <sup>d</sup> $\pm$ 0.10                  | 2.0 <sup>e</sup> $\pm$ 0.02                  | 1.1 <sup>d</sup> $\pm$ 0.06                  |
| RR | 198 <sup>d</sup> $\pm$ 14.5                                  | 1.2 <sup>e</sup> $\pm$ 0.08                  | 3.2 <sup>e</sup> $\pm$ 0.12                  | 1.7 <sup>d</sup> $\pm$ 0.02                  | 1.0 <sup>d</sup> $\pm$ 0.08                  |
| TL | 334 <sup>g</sup> $\pm$ 18.4                                  | 1.1 <sup>d</sup> $\pm$ 0.04                  | 4.6 <sup>f</sup> $\pm$ 0.17                  | 3.1 <sup>g</sup> $\pm$ 0.03                  | 1.6 <sup>g</sup> $\pm$ 0.09                  |
| TR | 132 <sup>b</sup> $\pm$ 4.4                                   | 0.8 <sup>b</sup> $\pm$ 0.07                  | 2.0 <sup>b</sup> $\pm$ 0.06                  | 1.1 <sup>b</sup> $\pm$ 0.02                  | 0.6 <sup>b</sup> $\pm$ 0.04                  |
| TF | 288 <sup>f</sup> $\pm$ 10.1                                  | 1.4 <sup>f</sup> $\pm$ 0.10                  | 4.4 <sup>f</sup> $\pm$ 0.17                  | 2.3 <sup>f</sup> $\pm$ 0.02                  | 1.2 <sup>e</sup> $\pm$ 0.06                  |
| LL | 295 <sup>f</sup> $\pm$ 10.1                                  | 1.3 <sup>e</sup> $\pm$ 0.09                  | 4.6 <sup>f</sup> $\pm$ 0.22                  | 2.3 <sup>f</sup> $\pm$ 0.02                  | 1.5 <sup>f</sup> $\pm$ 0.06                  |
| LR | 162 <sup>c</sup> $\pm$ 5.2                                   | 0.9 <sup>c</sup> $\pm$ 0.06                  | 2.6 <sup>c</sup> $\pm$ 0.07                  | 1.5 <sup>c</sup> $\pm$ 0.02                  | 0.9 <sup>c</sup> $\pm$ 0.07                  |
| LF | 52 <sup>a</sup> $\pm$ 2.1                                    | 0.3 <sup>a</sup> $\pm$ 0.01                  | 0.8 <sup>a</sup> $\pm$ 0.03                  | 0.4 <sup>a</sup> $\pm$ 0.01                  | 0.2 <sup>a</sup> $\pm$ 0.01                  |

Values with the same superscript letters are not significantly different ( $p < 0.05$ ) (mean for triplicate samples)

### Statistical Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) using Minitab software program. Differences at  $p < 0.05$  were considered statistically significant. The results were presented as mean values  $\pm$  SD (standard deviations). Measurements were carried out in triplicate for the antioxidant activity and quadruplicate for the emulsion system model.

Pearson's correlation was used to investigate the strength of the association between analysis of antioxidant activity and the total phenolic contents using Minitab software.

### HPLC Analysis of the Extracts

Before HPLC analysis, the ethanolic extract (1.5 mL) was filtered. Analyses were performed with Waters 2695 HPLC equipped with an autosampler (injection volume 20  $\mu$ L). The column was a Kinetex C18, 2.6  $\mu$ m, 100  $\times$  4.6 mm. The mobile phase was a mixture of solvent A (MilliQ water + 0.1 % glacial acetic acid) and solvent B (acetonitrile + 0.1 % glacial acetic acid) according to a linear gradient, lasting 12 min, changing from 85 % A to 70 % A in 8 min, at a flow rate of 1 mL/min. The detection was performed using a Waters 996 detector. Signals at a wavelength of 280 nm were stored and collected by Millennium software. The data are expressed as ppm of each phenolic compound.

## Results and Discussion

### Analysis of Total Polyphenols

The concentration of total polyphenols in the extracts was determined, and the results are shown in Table 1. The

extract from the leaves of thyme (*T. vulgaris*) had the highest polyphenol content with 334  $\pm$  18.4 mg gallic acid/g lyophilized extract, with the extract from thyme flowers and lavender (*L. angustifolia*) leaves next in polyphenol content with no significant difference between the samples ( $p = 0.751$ ). Lavender flowers had a lower polyphenol content (52  $\pm$  2.1 mg gallic acid/g lyophilized extract) than the leaves. The phenolic content of rosemary (*R. officinalis*) was in the range 198  $\pm$  14.5 and 219  $\pm$  6.1 mg GAE/g lyophilized extract. Several authors have reported the polyphenol content of this plant. Dorman et al. [10] reported a similar content in rosemary leaves (185 mg GAE/g extract), when determined following extraction with water.

However, studies involving methanol extraction of rosemary leaves have reported lower values than those obtained in the present study, in the range of 2.6–59.6 mg GAE/g dry weight (relative to the whole plant, not lyophilized), with 80 % methanol (MeOH:H<sub>2</sub>O) used as extracting solvent [11]. Extraction with 50 % ethanol (EtOH:H<sub>2</sub>O) allows higher recovery of the phenolic compounds [12]. Moreno et al. [13] also found a lower polyphenol content of 120 mg GAE/mL in a methanol extract.

Thyme is a rich source of polyphenols, especially in its leaves. Gramza-Michalowska et al. [14] reported a value of 229.63 mg GAE/g lyophilized powder after ethanol extraction. This value is lower than those found in the present study, 334  $\pm$  18.4 mg GAE/g lyophilized powder for leaves and 288  $\pm$  10.1 mg GAE/g lyophilized powder for flowers. These results are also higher than those reported for other species such as leaves of *T. argaeus*, which were reported to contain 83.31  $\pm$  0.59 mg GAE/g methanol extract [15].

The ethanolic extract of *L. officinalis* demonstrated the high phenolic content in this plant, particularly in the leaves (295  $\pm$  10.1 mg GAE/g lyophilized extract). The polyphenol content of extracts of this species extracted

with other solvents (like 70 % acetone) were lower (74 mg/g extract) [16]. Also, Miliuskas et al. [17] published a value of  $5.4 \pm 0.2$  mg GAE/g extract after extraction with methanolic solvents.

The solvent can have an important effect on the polyphenol content and antioxidant activity. Several procedures for extraction have been described proposing different times of extractions, solvents and weight/volume ratios, but organic extracts give higher yields of rosmarinic acid from herbs than extraction with water [18]. Extracts of rosemary in organic solvents have lower DPPH radical scavenging activity than aqueous extracts [19]. Miliuskas et al. [17] found that yields of extract from *L. angustifolia* with different solvents were in the order methanol > acetone > ethyl acetate, but the radical scavenging activity was relatively weak compared with other plant extracts, reflecting the low flavonoid content of the extracts.

Considering the co-presence of polar and nonpolar phenols, it was decided to extract with 50 % aqueous ethanol. Compositions of each extract are presented in Table 2.

The analysis showed that samples with higher rosmarinic acid and caffeic acid content generally had higher antioxidant activity. The highest content of rosmarinic acid was found in TL (396.2 mg/kg), TF (102.2 mg/kg), followed by RR (85.2 mg/kg) and LL (81.6 mg/kg). Rosmarinic acid possesses a broad spectrum of biological activities and is known as an antiviral, antibacterial, antioxidant, antiinflammatory and immunostimulating agent [20, 21]. Among the Lamiaceae species investigated by Janicsák et al. [22], thyme was an abundant source of rosmarinic acid, followed by lavender, which is in agreement with our findings.

Like rosmarinic acid, caffeic acid is also a common constituent of the plants of the Lamiaceae family. Caffeic acid was identified in extracts of TL (71.6 mg/kg), TR (169.2 mg/kg) and in LR (114.1 mg/kg). These results are in agreement with those of Janicsak et al. [22] who also

reported a lower content of caffeic acid than rosmarinic acid in the Lamiaceae plants.

Caffeic acid, luteolin, rosmarinic acid and hispidulin were present in a thyme extract [23], whereas caffeic acid, kaempferol and myricetin were present in the extract of lavender flowers [24]. Phenolic diterpenes (carnosic acid, carnosol, 12-*O*-methylcarnosic acid), caffeoyl derivatives (rosmarinic acid) and flavones (isoscuteallarein 7-*O*-glucoside and genkwanin) were identified in rosemary leaves, but the flavones and 12-*O*-methylcarnosic acid were absent from the stems [25].

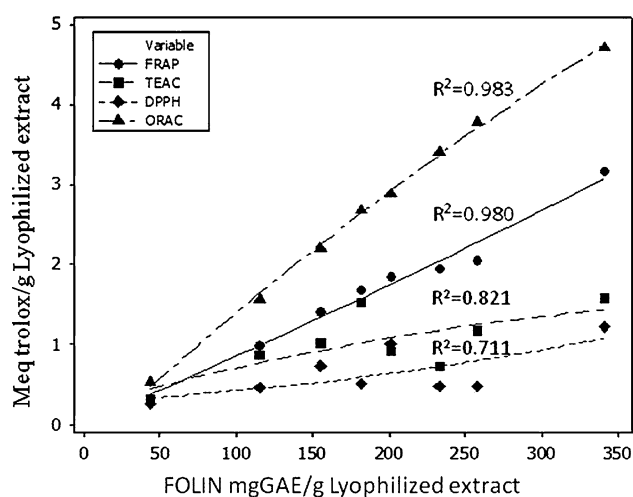
#### Antioxidant Activity

The antioxidant capacity of extracts from leaves, stems and flowers of rosemary, thyme and lavender was investigated by the TEAC, ORAC, DPPH and FRAP assays (Table 1).

The antioxidant capacity of the extracts was in the order LL, TL, TF > RR > RL > LR > TR > LF by the ORAC assay; TF > RR, LL > TL > LR > TR, RL > LF in the TEAC assay and TL > TF, LL > RL > RR > LR > TR > LF in the FRAP assay and TL > LL > TF > RL, RR > LR > TR > LF in the DPPH assay. This compares with the total phenol content in the order: TL > TF, LL > RL > RR > LR > TR > LF. For all 4 antioxidant assays, the TF, LL and TL extracts had the highest antioxidant capacity, with minor differences in the order of activity depending on the assay. This is consistent with the highest polyphenol content being present in these extracts. The LF and TR extracts had the lowest antioxidant capacity, and again this is consistent with their low polyphenol content. The RR, RL, and LR extracts were intermediate in polyphenol content and also in antioxidant capacity with minor differences in the order depending on the assay used. The main differences were in the ratio of antioxidant activity for RL and RR which had values of 0.91, 0.67, 1.18, 1.10 for the ORAC, TEAC, FRAP and DPPH assays, when the Folin–Ciocalteu assay indicated the ratio of polyphenols was 1.08. Thus the FRAP and DPPH values were consistent with the polyphenol content, whereas the ORAC and TEAC assays were more responsive to the polyphenols in RR. Phenolic diterpenes (carnosic acid, carnosol, 12-*O*-methylcarnosic acid), caffeoyl derivatives (rosmarinic acid) and flavones (isoscuteallarein 7-*O*-glucoside and genkwanin) were identified in rosemary leaves, but the flavones and 12-*O*-methylcarnosic acid were absent from the stems [25]. The presence of *o*-dihydroxy substituents in the aromatic ring of carnosic acid, carnosol and rosmarinic acid would suggest that these were more active antioxidant components than the flavones and 12-*O*-methylcarnosic acid, and this would be consistent with the increased activity of the stem extract in the ORAC and TEAC assays which depend on radical-scavenging activity.

**Table 2** Amount of rosmarinic acid and caffeic acid quantified by HPLC

| Extracts | Content of rosmarinic acid ppm (mg/L extract) | Content of caffeic acid ppm (mg/L extract) |
|----------|---|--|
| RL       | 15.14 ± 19                                    | 41.42 ± 51                                 |
| RR       | 156.61 ± 65                                   | 112.40 ± 51                                |
| TL       | 392.21 ± 1                                    | 179.65 ± 8                                 |
| TR       | 80.16 ± 12                                    | 67.00 ± 4                                  |
| TF       | 104.20 ± 3                                    | 82.27 ± 12                                 |
| LL       | 81.04 ± 1                                     | 113.61 ± 1                                 |
| LR       | 52.14 ± 3                                     | 55.99 ± 6                                  |
| LF       | 40.97 ± 1                                     | 29.08 ± 1                                  |



**Fig. 2** Plot of the association between the polyphenol content determined by the Folin–Ciocalteu analysis and antioxidant activity assessed by the FRAP, DPPH, TEAC and ORAC assays

The FRAP assay is a measure of reducing capacity and the DPPH assay is sensitive to steric hindrance, with different antioxidants reacting at different rates, and can give misleading results. DPPH is a stable nitrogen-centered radical, and many antioxidants do not react rapidly with DPPH because of steric hindrance due to bulky substituents in the chemical structure around the radical, which makes small antioxidant molecules generally show greater activity [26].

The polyphenol content assessed by the Folin–Ciocalteu assay correlated with antioxidant capacity assessed by the FRAP, TEAC and ORAC assays (Fig. 2). The correlation coefficients were  $r = 0.980$  (FRAP),  $r = 0.821$  (TEAC), and  $r = 0.983$  (ORAC).

In the case of the DPPH method the correlation was not so good ( $r = 0.711$ ). This may be partly due to reaction of the DPPH radical with components other than the polyphenols present in the solution, but steric hindrance probably also contributed.

There is extensive literature on the antioxidant capacity of herb and spice extracts. The values obtained by the TEAC assay for rosemary (leaves and stems) were  $0.8 \pm 0.04$  and  $1.2 \pm 0.08$  mmol Trolox equivalent/g lyophilized extract respectively. These values are much higher than the values of 0.274 and 0.324 mmol Trolox equivalent/g extract, reported by Tawaha et al. [27] for rosemary after extraction with 80 % methanol or hot water.

Tsai et al. [24] reported a TEAC value of 0.274 mmol/g for an extract from lavender flowers, which is similar to the value of 0.3 mmol Trolox equivalent/g lyophilized extract found in the current study. There are not many literature reports using the ORAC assay in the plants studied. Zheng and Wang [23] reported a value of 19.49  $\mu\text{mol/g}$  fresh weight of thyme. This value is lower than our study where the range is between 30 and 100  $\mu\text{mol}$  Trolox equivalent/g

fresh weight. Brewer [28] reported that the ORAC values for thyme, both fresh and dry, were 0.27 mmol Trolox equivalent/g fresh weight and 0.15 mmol Trolox equivalent/g dry weight, respectively. This value for dry thyme is much lower than those found in the current study, which has values between 0.3 for the stems and 0.6 mmol Trolox equivalent/g dry weight for the leaves. The extracts with the highest percentage inhibition by the DPPH assay were the leaves of rosemary and thyme, with values of 81.7 and 73.5 % respectively. These extracts contained components that reacted relatively slowly with the DPPH radical. The percentage inhibition during the 75 min of the experiment did not reach a limit and components continued to react with the radical after this time. The remainder of the extracts reached stability after about 70 min, presenting a faster kinetics.

Babovi et al. [15] reported that the DPPH radical scavenging activity of thyme leaves was greater than that of rosemary leaves, which agrees with this study.

Lavender showed a percentage inhibition (%) between 23 and 56 % in the DPPH assay. This compares with the percentage inhibition of 23 % for an ethanol extract [29], which is comparable to the values obtained in this study.

The strong antioxidant power of thyme and rosemary extracts measured by the FRAP assay was reported in the literature [30, 31]. The antioxidant power for extracts of rosemary and thyme, extracted with 80 % v/v methanol was 0.01 mmol Trolox equivalent/g dry extract for both. The recovery of polyphenolic compounds and the antioxidant capacity for extracts of the plants in our study in which 50 % aqueous ethanol was used for extraction were higher.

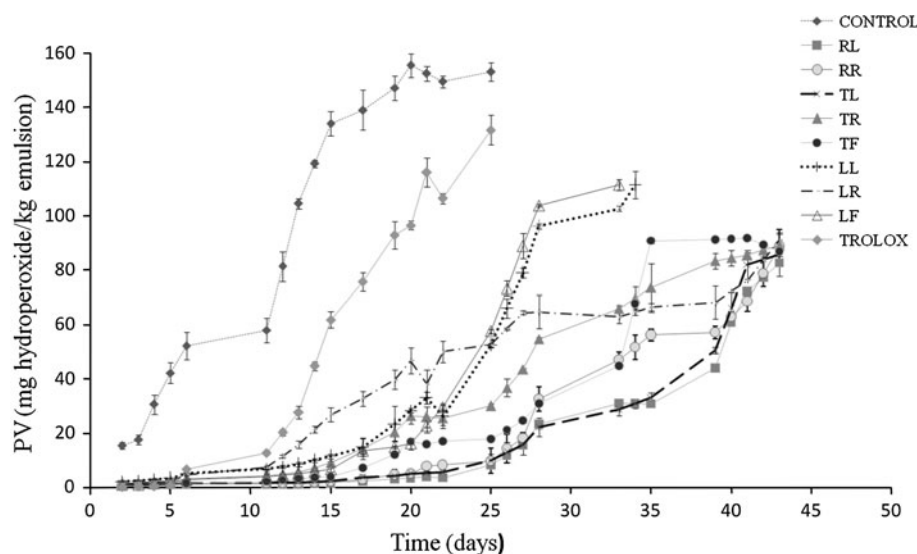
#### Antioxidant Effects in Stored Emulsions

In the study the antioxidant activity of rosemary, lavender and thyme extracts was analyzed in oil-in-water emulsions, as a model food (O/W) during 43 days. Each part of the plants was studied separately (leaves, flowers and stems). The oxidation was followed by assessment of the primary oxidation products (peroxide value) and the secondary oxidation products (TBARs value). In addition the change in pH was monitored, since pH tends to fall during oxidation.

#### Evolution of Peroxide Value

Figure 3 shows the evolution of PV versus time. The control (without extract added) was oxidized first, and the second sample oxidized was the positive control (Trolox 250 ppm). The sample containing Trolox, and the samples containing extracts were not significantly oxidized during the first 7–9 days. After this first period, the sample containing Trolox was oxidized more rapidly than the samples containing extracts. The time required for the emulsions to

**Fig. 3** Changes in peroxide value of emulsions during storage for 42 days



reach a peroxide value of 10 mequiv hydroperoxides/kg of emulsion was determined as a measure of stability. This value was taken since the limit for products of edible fats (animal, plant and anhydrous), margarine, and fat preparations, to guarantee quality is <10 mequiv hydroperoxides/kg. The first sample to reach 10 mequiv hydroperoxides/kg of emulsion was the control sample, and this occurred rapidly (2 days). The next samples to reach this level of deterioration were the positive control (Trolox) and LR. Other samples followed the order of stability:  $LF \approx LL \approx TR < TF < RR \approx TL \approx RL$ .

It is interesting to compare this order of stability with the results of the antioxidant assays where TF, LL and TL were most active followed by RR, RL, LR and then LF and TR were least active. There are clear differences in the activity in the homogeneous antioxidant assays and in the emulsion. This can be explained by the phenomenon known as the polar paradox, where antioxidants with less polar characteristics are more effective in oil-in-water emulsions than in homogeneous solution [32]. The reduced activity of TF and LL in the emulsion is consistent with the presence of more polar antioxidants in these extracts. Blazekovic et al. [33] reported that rosmarinic acid was a major polyphenol in an ethanolic extract of lavender leaves. It has previously been reported that rosmarinic acid is more active than carnosol and carnosic acid in oil but is a less effective antioxidant in an emulsion because of its relatively high water solubility [6]. The antioxidant activity of rosemary extracts is associated with the presence of various phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, and rosmarinic diphenol rosmariquinone. Del Bano et al. [25] and Frankel et al. [6] reported that the carnosol and carnosic acid content was higher than the rosmarinic acid content in rosemary leaves. Carnosic acid has been described as a lipophilic antioxidant that

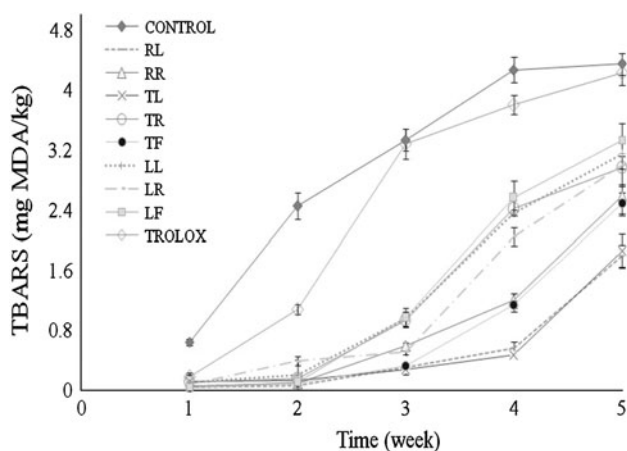
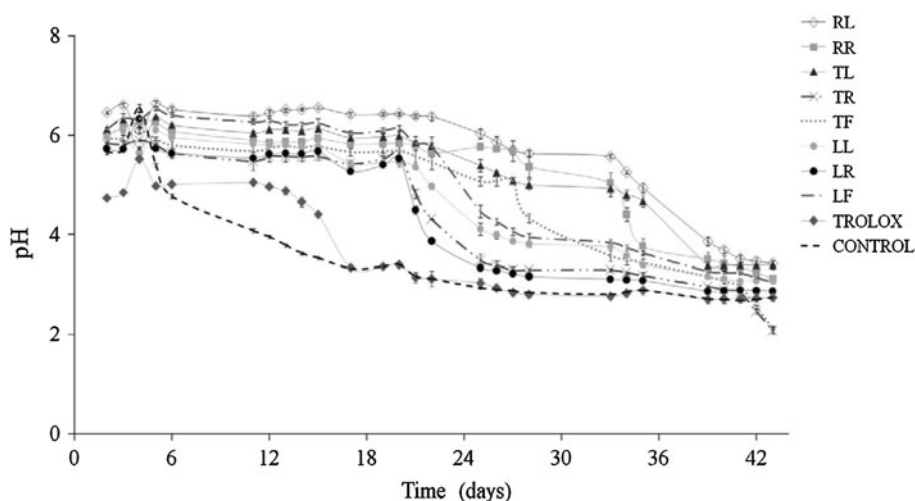
scavenges hydroxyl radicals and peroxy radicals, preventing lipid peroxidation [34]. Hence, the carnosol, and carnosic acid in RR and RL help the extracts to retain their antioxidant properties in the emulsion whereas the TF, which relies more on its rosmarinic acid content for its antioxidant effectiveness in the homogeneous antioxidant assays, becomes less effective in the emulsion. Ferulic and gallic acids were also reported to be major antioxidants in extracts from the flowers of the related species *T. capitata* and these phenolic acids are known to be polar, and hence if these were also present in thyme (*T. vulgaris*), they would be less effective in an emulsion [35].

#### Evolution of pH Over Time

The pH of the samples was measured as a parameter to investigate its correlation with PV, since some hydroperoxide decomposition products are acidic (Fig. 4). The pH fell as the PV increased. Many antioxidants have the disadvantage of being less effective as antioxidants when the pH is low. A significant effect of pH on the antioxidant activity of carnosic acid and carnosol (main components of rosemary) was reported with a high antioxidant activity at pH 4–5 [6]. Lipid oxidation in emulsions is slower at higher pH and the speed is accelerated as pH decreases [6].

Following the order of primary oxidation, the pH experienced a decline in accordance with oxidation. All samples started with a near neutral pH. The negative control (without extract) and positive control (Trolox) were the first to change. The control sample had a marked decrease in pH from day 6 and the Trolox from day 14. The lavender extract was oxidized more rapidly with a fall in pH detectable from 20 days and the pH decreased in the order  $LR > LL > LF$ . Parts of thyme (leaves and stems) had a similar behavior to the other extracts until



**Fig. 4** pH evolution during 42 days of study**Fig. 5** Changes in TBARS values during the study

time = 20 days, when the pH decreased for TR, and day 27 when it decreased for TF. The stability of samples containing RR, RL and TL was similar. There was a reduction between 33 and 34 days, but at this time the pH of the RL was higher than that of the others.

#### Evolution of TBARS

Hydroperoxides decompose to form secondary oxidation products, which are responsible for the flavor, the rancid odor and undesirable taste of oxidized fats [36].

Secondary oxidation products were monitored by measurement of the TBARS (Fig. 5). According to analysis after 5 weeks, TBARS values of emulsions containing added herb extracts were lower than that of the control (4.35 mg MDA/kg) and the Trolox (4.23 mg MDA/kg). The TBARS value increased from 3 weeks. RL (1.79 mg

MDA/kg) and TL (2.50 mg MDA/kg) were the most effective antioxidants, followed by TF (2.50 mg MDA/kg) and RR (2.59 mg MDA/kg). The highest TBARS value for the herbs, were for LR, TR, LL and LF.

It can be concluded that RL and TL had the best antioxidant effect based on findings from the PV and TBAR measurements.

#### Conclusions

This study proved the antioxidant activity of extracts from the aromatic plants. The results obtained showed that there are significant differences between different parts of the plants studied. The highest content of total polyphenols and antioxidant capacity assessed by homogeneous antioxidant assays was in the extracts from thyme flowers, lavender leaves and thyme leaves, followed by the stems and leaves of rosemary. The sample with the lowest concentration of polyphenols and lowest antiradical capacity was the lavender flower extract.

Rosemary leaves and thyme leaves were most effective at protecting the emulsion against oxidation followed by the rosemary stems and thyme flowers. No significant differences between the leaves of thyme and rosemary were detected.

This research has demonstrated the antioxidant properties of the plant tissues of these species, and they may represent an alternative to synthetic antioxidants in preservation of food, as well as in the pharmaceutical industry and cosmetics.

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