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Exploitation of the biological potential of *Satureja thymbra* essential oil and distillation by-products

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ABSTRACT

Dried leaves of *Satureja Thymbra* were subjected to water-steam distillation to recover the essential oil (EO) and the water extract (Aq). The solid by-product was subjected to sequential Soxhlet extraction with ethyl acetate (EAc) and ethanol (EtOH). The obtained extracts were analysed by HPLC-DAD-ESI–MS/MS and the main flavonoids and phenolic acids were identified and quantified. The high antiradical activity of the extracts, as estimated by the DPPH• assay, was correlated to their total phenol content. Moreover, the antioxidant activity of the extracts was tested in bulk palm oil and palm oil-in-water emulsions. EAc extract prolonged the induction period and reduced by 42% the rate of peroxide formation in palm oil, while EtOH extract was the most effective in emulsions. Additionally, the EtOH and EAc extracts depressed the growth of *Listeria monocytogenes* in emulsions deliberately spiked with 100 cfu/mL.

The antimicrobial properties of EO, Aq, EAc, and EtOH extracts were assayed and the minimum inhibitory and non-inhibitory concentration values were determined. EO was effective against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Pseudomonas fragi*, *Saccharomyces cerevisiae* and *Aspergillus niger*. In contrast, EAc and EtOH extracts were active only against the bacteria species, but not against *S. cerevisiae* and *A. niger*, while no antimicrobial activity was observed for Aq extract.

1. Introduction

Currently, there is a strong debate and interest regarding the safety aspects of chemical preservatives added widely in many food products to prevent mainly growth of spoilage and pathogenic microbes. The synthetic compounds are considered responsible for many carcinogenic and teratogenic attributes and residual toxicity. To remedy the aforementioned problems, consumers and the European authorities increased the pressure on food manufacturers to substitute the harmful artificial additives with alternative natural substances. In this context, the use of natural compounds with antimicrobial activity presents an intriguing case. Moreover, natural compounds, like polyphenols, exhibit antiradical and antioxidant activities, and a certain body of research has been focused on their potential use in lipid food to retard oxidation.

Herbs of the Lamiaceae family are well known raw materials that contain substances with bioactive, antimicrobial, or antioxidant properties. The current commercial exploitation of these herbs is limited to the recovery of the essential oil, while the remaining solid waste is discarded, often creating an adverse environmental impact. Among the *Lamiaceae* herbs, *Satureja thymbra* is wildly grown and also cultivated in Mediterranean counties. The essential oil of the herb has been analysed and tested for antimicrobial activity by several researchers (Gören et al., 2004; Fleisher and Fleisher 2005; Chorianopoulos et al., 2006b; Karabay-Yavasoglu et al., 2006; Giweli et al., 2012; Öztürk, 2012; Tepe and Cilkiz, 2015). However, the essential oil consists a minor fraction of the plant, while the solid waste remaining after the recovery of essential oil contains polyphenolic compounds that could be used as antimicrobial or antioxidant agents. Skoula and Grayer (2005) detected some flavonoids in the aerial part of the herb, while there is no literature report about a systematic analysis of other phenolic compounds.

Abbreviations: Aq, water extract; EAc, ethyl acetate extract; EO, essential oil; EtOH, ethanol extract.

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Flavonoids, flavonoid glycosides and phenolic acids may be fractionated and recovered from aromatic plants by successive extractions with organic solvents. Research on the component characterization, the antimicrobial and antioxidant properties of *S. thymbra* extracts rich in polyphenolic substances is rather limited. Therefore, the present study focused on recovery of the essential oil and the polyphenolic fractions of *S. thymbra,* analysis of their components and determination of the antimicrobial, antiradical, and antioxidant properties. The obtained results might help in exploitation of the herb, and further its use beyond the essential oil recovery.

2. Materials and methods

2.1. Reagents and standards

Dried leaves of *Satureja thymbra* were obtained from the Institute of Plant Breeding and Genetic Resources" – Hellenic Agricultural Organization DEMETER. The plant was cultivated in the experimental field of the Institute, harvested in early May 2014, and provided to our Laboratory just after drying. The extractions were performed with ethyl acetate (Fisher Scientific, Loughborough, UK) and ethanol 96°. The reagents included DPPH (Sigma-Aldrich, Steinheim, Germany), Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and sodium carbonate (Mallinckrodt, St. Louis, Missuri). The standard compounds used in the study were quercetin dihydrate and rosmarinic acid, products of Sigma-Aldrich (Steimheim, Germany), as well as luteolin, apigenin, eriodictyol, and naringenin (Extrasynthese, Genay, France), carvacrol (≥97%, Merck, Darmstadt, Germany), γ-terpinene (≥97%, Fluka, St Louis, USA) and gallic acid (98% w/w, Acros Organics, Fair Lawn, New Jersey). Water, acetonitrile, and methanol were used for chromatography analyses (HPLC and MS grade) (Fisher Scientific, Loughborough, UK), while glacial acetic acid (HPLC grade) (PanReac, Barcelona, Spain) was used for the acidification of HPLC solvents.

2.2. Microbial strains

Salmonella enterica subsp. *enterica* ser. Enteritidis FMCC B56 PT4 (kindly provided by Prof. Nychas G.J.E., Agricultural University of Athens, Greece), *Salmonella enterica* subsp. *enterica* ser. Typhimurium DSMZ 554, *Listeria monocytegenes* NCTC 10527 serotype 4b, *Escherichia coli* ATCC 25922, *Staphylocccus epidermidis* FMCC B-202 C5M6 (kindly provided by Dr. Nisiotou A., Athens Wine Institute, ELGO-DIMITRA, Greece), *Staphylococcus aureus* ATCC 25923, *Pseudomonas fragi* 211 (kindly provided by Prof. Nychas G.J.E.), *Saccharomyces cerevisiae* uvaferm NEM (Lallemand, Canada) and *Aspergillus niger* 19111 (kindly provided by Prof. Nychas G.J.E.) were used in the present study.

2.3. Extraction procedure

S. thymbra was subjected to a series of processes for the recovery of the bioactive compounds. The flow diagram of Fig. 1 depicts the sequence of treatments, the obtained products and their coded names. Dry leaves (500 g) of the plant were initially subjected to water-steam distillation to recover the essential oil (EO) and the water extract (Aq). An open type pilot scale distiller, made of copper (Chalkos, Greece) was used for the distillation. The apparatus was equipped with inner perforated grid to hold the plant material (net vol. for plant 10 L) above the boiling water (3.5 L). The headspace between the level of boiling water and the plant material was 3.5 L at the start of distillation, while the distillation procedure was carried for 3 h. The distilled water (hydrosol or floral water) was not recycled and, therefore, it was collected together with the EO. It amounted to 460 mL and was not further studied. The EO was kept in sealed glass vial in the refrigerator until used. Water-soluble compounds of the herb were partially extracted by the condensed steam and accumulated in the boiling water of the distiller. The remaining water phase at the end of distillation amounted to approximately 1.9 L. It was collected, filtered, diluted to 2 L and comprised the Aq. Aq was stored in a plastic container at the refrigerator, until used.

The wet herbal residue (~1.6 kg) was further dried, in a ventilated oven (Function Line UT20, Heraeus Instruments GmbH, Hanau, Germany) at 35 °C for 24 h, ground in a laboratory mill (Retch ZM 1; Haan, Germany), equipped with a 0.5 mm sieve, divided to batches of 50 g, and subjected to Soxhlet extractions sequentially with ethyl acetate and ethanol to obtain the EAc and

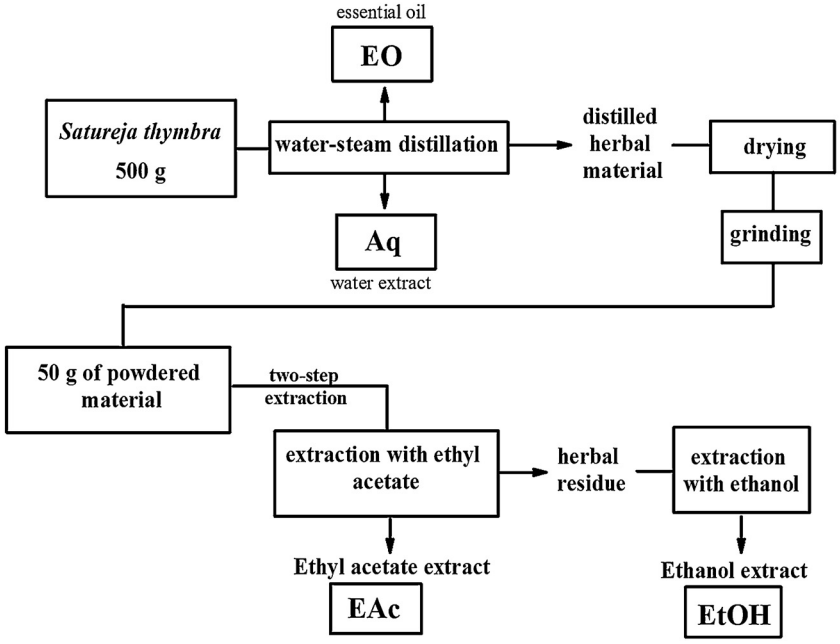

Fig. 1. The flow diagram of the extraction procedure and the coded names of the essential oil (EO), water (Aq), ethyl acetate (EAc) and ethanol (EtOH) extracts obtained from *S. thymbra*.

EtOH, respectively. The extractions were carried for 6–8 h, under mild reflux, until the extract was colorless, indicating the absence of phenolic compounds that impart a yellow coloration. The extracts were filtered, transferred to 500 mL volumetric flasks, made up to the volume, and kept in a refrigerator (<4 °C) until further used.

The whole procedure was repeated twice and the presented results are mean values.

2.4. Analytical procedures

2.4.1. Determination of phenolic composition by HPLC-DAD–ESI-MS/MS

The identification of phenolic compounds was performed on a Varian 212-LC chromatography system, coupled to an ion trap mass spectrometer equipped with an electrospray interface, and a diode array detector, as described by Choulitoudi et al. (2016). Briefly, samples were injected after filtration (0.45 μm, PVDF syringe filters, Teknokroma, Barcelona, Spain) on a reversed phase Hypersil C18 column (ODS 5 μm, 250 × 4.6 mm, MZ Analysentechnik, Mainz, Germany). Solvent A: water, and solvent B: methanol/acetonitrile 60/40, both acidified with acetic acid (1%) were used, with the following linear gradient: 0 min: 90% A, 35 min: 71% A, and 70 min: 0% A. The flow rate was maintained at 0.4 mL/min, the injection volume was 20 μL and the DAD detection was performed at 280 nm. Additional wavelengths were applied for the detection of flavonols and flavones (360 nm), and hydroxycinnamic acids (320 nm). The parameters employed for ESI-MS/MS were: negative ionization mode; drying gas, N_2; drying temperature, 350 °C; nebulizer, 65 psi; capillary, 3.5 kV; compound stability, 100%; scan range, 100–1200 *m/z*. System control and data acquisition was performed using the Varian Workstation software (Varian Inc., Palo Alto, California). The identification of the components was based on UV–vis spectra, retention times, and mass fractions, while literature data were used for comparison when standards were not available.

The quantification of individual compounds was based on the respective reference curves constructed for eriodictyol, quercetin, rosmarinic acid, and carvacrol. In terms of comparison the EO was also analysed by HPLC-DAD for the quantification of carvacrol.

2.4.2. Total phenol content (TP)

The Folin Ciocalteau method (Waterhouse, 2005) was used for the quantification of total phenols (TP) of the extracts. The results were expressed in gallic acid equivalents (GAE), through construction of a reference curve. All samples were analysed in duplicate and the presented results are mean values of duplicate experiments.

2.4.3. Antiradical activity against DPPH•

The antiradical activity was determined against DPPH according to the method proposed by Brand-Williams et al. (1995), under thermostated conditions at 25 °C. Duplicate experiments for each dilution were performed and the% remaining DPPH was calculated and plotted against $g_{extract}/kg_{DPPH}$ to estimate the EC_{50} parameter through linear regression. The same procedure was used to determine the EC_{50} parameter of standard compounds (rosmarinic acid, carvacrol and γ-terpinene).

2.4.4. Antioxidant activity in bulk oil

The antioxidant activity of the extracts was examined against palm oil oxidation. Palm oil (110 g, AGROTIKI SA, Athens, Greece) was weighed in a spherical flask, and the proper volume of extract was added under continuous stirring, to obtain a concentration of 500 ppm dry extract in oil. Subsequently, the solvent was removed in a vacuum evaporator (Hei-Vap Value Digital, equipped with vacuum pump Rotavac Valve Tec, Heidolph, Schwabach, Germany), followed by purging with nitrogen. Duplicate samples of 50 g from each oil solution, as well as pure palm oil, were transferred to 100 mL beakers, and subjected to accelerated thermal oxidation at 70 °C in a ventilated oven (Heraeus Instruments GmbH, Hanau, Germany). Samples were removed at definite time intervals and the oxidation was monitored by the determination of peroxide value (PV) according to the IUPAC method, 2.501 (IUPAC, 1987). Measurements were performed in duplicate, averaged, and the presented results are mean values of duplicate samples.

2.4.5. Antioxidant activity in emulsions

Oil-in-water emulsions were prepared with palm oil (AGROTIKI SA, Athens, Greece) at a concentration of 30% (w/w), with Tween 20 (2%, v/v) as emulsifier. The pH of the emulsions was adjusted to 6.8 using phosphate buffer 0.2 M. An appropriate volume of each extract was added to obtain a final concentration of 500 ppm dry extract in oil, as follows: EAc and EtOH were thoroughly mixed, under stirring, with palm oil, before the emulsification, and the organic solvents were removed as described above. The Aq was added to the water phase of the emulsion.

Pre-emulsions were prepared in a high speed homogenizer (CAT Unidrive 1000d, Paso Robles, California) at 5000 rpm, for 15 min. Then the pre-emulsions passed twice through a high-pressure homogenizer (APV SYSTEMS Albertslund, Denmark) operating in two stages (1st stage: 400 bar, 2nd stage: 20 bar). Samples (10 mL) were transferred in glass vials and placed in a ventilated oven (Heraeus Instruments GmbH, Hanau, Germany) at 70 °C for accelerated oxidation. To recover the oil, the samples were frozen at −28 °C for 24 h, thawed in a water bath at 60 °C for 20 min, and centrifuged (ThermoFisher scientific, Osterode, Germany) for 10 min, at 10000 rpm and 20 °C.

Primary and secondary oxidation products were measured in the oil recovered from the emulsion samples. Peroxide Value (PV) was determined according to the standard method of IUPAC, 2.501 (IUPAC, 1987), and conjugated dienes (CD), according to the standard method of IUPAC 2.205 (IUPAC, 1987). Secondary oxidation products, specifically *p*-anisidine value (*p*-AV), was measured according to the standard method of AOCS, Cd 18–90 (AOCS, 1994). All measurements were performed in duplicate, averaged, and the presented results are mean values of duplicate samples.

2.4.6. Antimicrobial activity

2.4.6.1. Microbial strains growth. *S. Enteritidis*, *S. Typhimurium*, *L. monocytegenes*, *E. coli*, *S. epidermidis* and *S. aureus* were grown in Brain Heart Infusion (BHI) broth (LABM, UK) at 37 °C for 24 h. Likewise, *P. fragi* was grown in Brain Heart Infusion (BHI) broth (LABM, UK) at 25 °C for 24 h. *S. cerevisiae* was grown in YPD broth (yeast extract 10 g/L, glucose 20 g/L and peptone 20 g/L) at 28 °C for 3 days. *A. niger* was grown on Malt extract agar (LABM) for 7 days at 37 °C.

2.4.6.2. Disk diffusion assay. For the antibacterial screening, the disk diffusion assay was performed. The bacterial suspensions were diluted 10-fold in ¼ Ringer's solution (LABM, UK). A 0.1 mL portion from the appropriate dilution was spread on Brain Heart Infusion (BHI) agar (LABM), in order to provide initial inoculums of 10^5 or 10^7 cfu/mL. Subsequently, sterile paper disks (Whatman no. 2) of 5 mm diameter were placed onto the inoculated agar surface containing 5 μL of the EO or the extracts. Petri dishes were incubated at 25 °C for *P. fragi* and at 37 °C for 24 h for the rest bacteria species. After incubation, the inhibition zones were measured in mm.

The same procedure was also followed for screening of the activity against yeasts, using *S. cerevisiae* suspensions spread on YPD agar, while the petri dishes were incubated at 28 °C for 3 days. For the antifungal activity, 100 fungal spores/plate from *A. niger* were spread on Malt Extract agar (LABM). The petri dishes were incubated at 37 °C for 10 days, and the inhibition zones were measured daily.

Water, ethyl acetate or ethanol were used as negative controls, while all experiments were carried out at least in triplicate, and the mean values are presented.

2.4.6.3. Determination of minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC). Determination of MIC and NIC values was carried out as recently described (Mitropoulou et al., 2015). In brief, bacterial growth in BHI broth (LABM) was monitored through changes in optical density of bacterial suspensions in the presence of multiple concentrations of the EO or the extracts. Stock solutions were prepared by mixing the appropriate amount of the EO or the extracts directly with BHI broth. Aliquots (0.180 mL) of growth medium mixed with the EO or the extracts were transferred to the wells of a 96-well microplate. The bacterial suspensions were diluted tenfold in ¼ Ringer's solution and a 0.070 mL portion from the appropriate dilution was added to the wells containing the growth medium (final volume 0.250 mL), in order to result in a population of approximately 10^3 cfu/mL. Microplates were incubated in Microplate Reader (Molecular Devices, VERSAmax, USA, Softmaxpro v. 5.0 software) at 25 °C for 24 h for *P. fragi* and at 37 °C for 24 h for the rest bacteria species. Optical density measurements were carried out every 10 min at 610 nm. BHI broths with no inoculum and inoculated BHI broths with the respective volume of water, ethyl acetate or ethanol were used as controls.

The calculation of MIC and NIC values was based on the Lambert-Pearson model (LPM) (Lambert and Lambert, 2003; Chorianopoulos et al., 2006a). In brief, the effect on the growth, measured by the optical density method, is manifested by a reduction in the area under the OD/time or curve relative to a control well at any specified time. By calculating the area using the trapezoidal rule, the relative amount of growth was obtained using the ratio of the test area to that of the control, called the fractional area, fa. Data were fitted to the LPM using non-linear least squares regression analysis assuming equal variance. Each experiment was performed at least 4 times and standard deviation was calculated by Fig P.2 .1 software (Fig.P Software Incorporated, Canada).

2.4.6.4. Antimicrobial activity in emulsions. The emulsions containing the Aq, EAc or EtOH of *S. thymbra* were deliberately spiked with *L. monocytogenes* culture in order to obtain an initial level of 10^2 cfu/ml viable cells and then stored in sterile bags, at 4 °C. At various intervals, samples were collected to monitor the levels of the inoculated strain. Therefore, representative, 25 g portions were blended with 225 mL of sterilized ¼ Ringer's solution in a stomacher (Lab Blender 400, Seward Medical, London) for 60 s at room temperature and subjected to serial dilutions. *L. monocytogenes* counts were determined in Palcam Agar (LABM) containing Palcam Listeria Selective Supplement (LABM) after incubation at 37 °C for 24–48 h. For comparison reasons, control samples with no addition of *S. thymbra* extracts were used. All experiments were carried out at in triplicate.

2.5. Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range test were applied to detect differences among emulsion samples containing the extracts in terms of PV, *p*-AV, totox value and microbial counts. EC_{50} values of the extracts were correlated to TP and total phenolic acids concentration and Pearson's correlation coefficients were determined. Analyses were performed with the STATISTICA software (version 10, StatSoft® Inc., United States). Differences were considered to be significant at $p < 0.05$. Moreover, linear regression analysis was used to determine the ratio of the PV increase in bulk oil, and was performed with SigmaPlot software (version 11.0, Systat® Inc., Germany).

3. Results and discussion

3.1. Yield of distillation and extraction processes

Satureja thymbra was treated according to the sequential processes presented in Fig. 1, and the yields of the obtained extracts, shown in Table 1, are expressed on dry basis of the initial herb. The EO amounted to 4.6 ± 0.3% (v/w dry herb). Similar yields have been reported by Giweli et al. (2012), Öztürk (2012), Economou et al. (2011), and Skoula and Grayer (2005).

During the distillation process, a partial extraction of water-soluble components in the boiling water at the bottom of the distiller occurred, and was collected as Aq extract that amounted to 45 g dry extract/kg dry herb. In the following Soxhlet extractions, ethanol recovered most of the herb components (116 g/kg dry herb), while less than half of the above mentioned yield was obtained with ethyl acetate (47 g/kg dry plant). The sum of the extractable material amounted to approximately 200 g/kg dry herb, showing a good yield of compounds with potential biological activity, in addition to the EO. Taking into consideration also the EO yield, the one quarter of the plant mass consists of recoverable compounds.

3.2. Consistency of S. thymbra EO and extracts

The analysis of EO has been presented in another work (Choulitoudi et al., 2016). The main component is γ-terpinene, amounting to 40.1% of total peak area, followed by carvacrol (30.8%), and, at a much lower consistency, *p*-cymene (9.3%), and *trans*-caryophyllene (7.6%). Minor components comprise α-terpinene (3.6%), α-thujene (1.8%), β-myrcene (1.3%) and anisole (1.2%), while the rest eleven compounds, α-pinene (0.9%), limonene (0.6%), β-pinene (0.4%), α-humulene (0.3%), α-phellandrene (0.3%), (−)-linalool (0.3%), camphene (0.2%), 4-terpineol (0.2%), borneol (0.1%), caryophyllene oxide (0.1%) and thymol (0.1%) are characterized as traces. The low consistency of thymol indicated that the herb used in this work belongs to the carvacrol chemotype. Similarly low or zero thymol contents have been reported by several researchers (Azaz et al., 2005; Fleisher and Fleisher 2005; Karousou et al., 2005; Glamočlija et al., 2006; Economou et al., 2011). Moreover, the high consistency in γ-terpinene is probably related to the early harvesting season of the plant (beginning of May), while a high γ-terpinene content was also reported by other researchers (Karabay-Yavasoglu et al., 2006; Skoula and Grayer, 2005).

The HPLC-DAD–ESI-MS/MS analysis of extracts revealed 6–8 main peaks in each extract and several minor ones. All the components identified in Aq, EAc and EtOH are presented in Table 2, while the quantification of the main flavonoid subgroups and phenolic acids of each extract is summarised in Table 1. EAc presented four major peaks that were identified as the flavones luteolin and apigenin, and the flavanones eriodictyol and naringenin, through comparison with the respective standard compounds. All four compounds were also detected by Skoula and Grayer (2005), who also reported the presence of aromadendrin and quercetin that were found in traces in our extract. 6-OH-Luteolin was present in the form of 7,3′-dimethyl and 7,3′,4′-trimethyl ethers, as identified according to their UV and mass spectra. These compounds have also been identified by Skoula and Grayer (2005) in *S. thymbra*. Additionally, eight minor peaks of the EAc presented the characteristic spectra of flavones/flavonols, and consequently were quantified in the respective subgroup in Table 1, while one minor peak was quantified in the dihydroflavonol/flavanone subgroup according to its spectrum.

The main flavonoids identified in EtOH were luteolin 7-*O*-rutinoside, apigenin 6,8-di-*C*-glycoside, apigenin and 6-OH-luteolin 7,3′-dimethyl ether. Luteolin 7-*O*-rutinoside presented a

Table 1
The distillation and extraction yields of *Satureja thymbra*, the quantification of the main groups of compounds on dry extract or distillate basis, and the activity against the DPPH radical.

Extract	Yield[a] (g/kg)	carvacrol (g/kg)	Total phenolic acids[c] (g/kg)	Total flavones/flavonols[d] (g/kg)	Total flavanones/ dihydroflavonols[e] (g/kg)	Total Phenols (g/kg GAE)	EC_{50} $\left(\frac{g_{antiox.}}{kg_{DPPH}}\right)$
EO	46 ± 3[b]	343.7 ± 14.0	n.d.	n.d.	n.d.	250.3 ± 3.3	1799
Aq	45 ± 1	3.4 ± 0.2	107.6 ± 1.2	63.1 ± 1.1	3.3 ± 0.1	249.5 ± 0.9	267
EAc	47 ± 1	17.0 ± 1.6	45.4 ± 1.1	87.2 ± 4.9	10.8 ± 0.2	154.0 ± 11.9	707
EtOH	116 ± 1	n.d.	210.8 ± 2.2	198.4 ± 3.8	0.7 ± 0.1	289.3 ± 6.2	222

[a] On dry plant basis.
[b] % mL/kg.
[c] Expressed as rosmarinic acid equivalents (apart from caffeic acid).
[d] Including glycosides and expressed as quercetin equivalents.
[e] Including glycosides and expressed as eriodictyol equivalents.

Table 2
The identified phenolic compounds of *S. thymbra* extracts.

	Rt (min)	Spectral Maxima (nm)	$[M-H]^-$ and main fragments	Aq	EAc	EtOH
compounds				concentration in dried extract basis (g/kg)		
caffeic acid	19.4	291, 323	179, 135	1.75 ± 0.01	–	2.69 ± 0.1
apigenin 6,8-di-C-glycoside	27.1	270, 338	593, 473, 383	11.0 ± 0.1	–	23.7 ± 0.2
luteolin 7,4′-di-O-glucuronide	34.2	257, 264sh, 346	637, 285	3.43 ± 0.02	–	2.44 ± 0.39
luteolin 7-O-rutinoside	43.5	254, 268sh, 348	593, 285	20.9 ± 0.70	–	57.0 ± 0.7
aromadendrin	45.6	290, 334sh	287	–	traces	–
rosmarinic acid	46.9	294sh, 330	359, 161	62.0 ± 0.60	24.6 ± 0.5	133.7 ± 1.2
apigenin 7-O-glycoside	47.8	337, 266	431, 269	3.10 ± 0.58	1.24 ± 0.25	10.2 ± 2.4
eriodictyol	49.0	290, 334sh	287	3.28 ± 0.12	6.7 ± 0.2	0.68 ± 0.03
unidentified phenolic acid	49.9	292, 326sh	717, 493, 359	9.09 ± 0.20	–	traces
lithospermic acid	50.4	254, 286, 310, 336sh	537, 493, 359	13.6 ± 0.30	–	8.0 ± 0.8
salvianolic acid A	51.3	287, 310sh, 338sh	493, 295	21.1 ± 1.00	20.8 ± 0.9	66.4 ± 1.7
naringenin[a]	53.1	290, 334sh	271	–	4.5 ± 0.3	–
quercetin	53.5	254, 371	301	–	2.22 ± 0.30	traces
luteolin[b]	54.7	254, 267sh, 350	285	–	10.5 ± 1.6	traces
apigenin[b]	57.2	268, 338	269	2.81 ± 0.27	5.4 ± 1.2	28.4 ± 0.5
carvacrol	60.0	220sh, 274	–	0.21 ± 0.12	17.0 ± 1.6	–
6-OH luteolin 7,3′-dimethyl ether[b]	60.8	286, 346	329, 314	–	30.8 ± 3.3	21.7 ± 1.2
6-OH luteolin 7,3′,4′-trimethyl ether[b]	64	251, 280sh, 343	327, 298	–	15.2 ± 2.6	7.1 ± 0.6

[a] Flavanones and dihydroflavonols, expressed as eriodictyol equivalents.
[b] Flavones and flavonols, expressed as quercetin equivalents.

pseudo molecular ion $[M-H]^-$ at m/z 593 that fragmented to m/z 285 that corresponds to the luteolin aglycone. The loss of 308 amu reflects the rutinosyl unit. Finally the UV–vis spectrum of the compound matches the pattern reported by Mabry et al. (1970). The compound has been identified in *Satureja hortensis* by Kemertelidze et al. (2004) and *Satureja montana* by López-Cobo et al. (2014). Apigenin 6,8-di-C-glycoside presented a flavone-type UV–vis spectrum and a pseudo molecular ion $[M-H]^-$ at m/z 593 which produced two main fragments at m/z 473 and 383. Martins et al. (2014), analyzing extracts from *O. vulgare* by LC–MS/MS, reported an identical fragmentation pattern for $[M-H]^-$ at m/z 593 and identified the compound as apigenin 6,8-di-C-glycoside. According to them, the above fragments correspond to the loss of 120 and 90 amu characteristic of C-hexosyl flavones, and at m/z 353 that would correspond to the apigenin aglycone bearing some sugar residues [apigenin + 83 mu]. Apigenin 6,8-di-C-glycoside $[M-H]^- = 593$, with similar fragmentation and UV–vis spectrum has also been identified in *Origanum vulgare* L. ssp. *hirtum* by Grevsen et al. (2009) and Koukoulitsa et al. (2006).

Moreover, 3 minor peaks were identified as luteolin 7,4′-di-O-glucuronide, apigenin 7-O-glycoside and 6-OH luteolin 7,3′,4′-trimethyl ether, while eriodictyol was detected in trace quantity. Luteolin 7,4′-di-O-glucuronide presented an $[M-H]^-$ at $m/z = 637$ amu, which fragmented to $m/z = 285$ amu (luteolin) and the loss of 352 amu corresponds to two glucuronyl moieties. The compound was tentatively identified as luteolin 7,4′-di-O-glucuronide, since both UV–vis and MS spectra were similar to the ones identified by Yin et al. (2012) and Grevsen et al. (2009) for the respective compound in *Origanum vulgare* L. ssp. *hirtum*. The compound with $[M-H]^-$ at $m/z = 431$ amu was identified as apigenin glycoside since the fragmentation produced an ion at $m/z = 269$ (apigenin) and the loss of 162 amu corresponds to one hexosyl moiety. Apigenin 7-O-glycoside is a common glycoside in *Lamiaceae*, which has been identified by numerous researchers (López-Cobo et al., 2014; Yin et al., 2012; Grevsen et al., 2009; Koukoulitsa et al., 2006; Koşar et al., 2005).

EtOH contained six more flavonoid peaks, which according to their UV–vis spectra, were categorized to the flavone subgroup. The compounds could not be identified, however they were included in the subgroup of total flavones/flavonols (Table 1).

Aq contained luteolin 7-rutinoside and apigenin 6,8-di-C-glycoside as main flavonoids, while luteolin 7,4′-di-O-glucuronide, apigenin 7-O-glycoside, eriodictyol and apigenin were also identified as minor flavonoids. The spectral data of the above compounds matched the identifications already performed for the previous extracts. As can be seen in Table 1, total flavones/flavonols predominated in all extracts, and especially in EtOH, compared to flavanones/dihydroflavonols.

Rosmarinic acid was the main phenolic acid in all extracts, followed by salvianolic acid A, and lithospermic acid. Rosmarinic acid was identified with the use of internal standard, UV–vis and MS spectra, while the structure of the two other phenolic acids was confirmed with comparison of chromatographic and spectral data with other researchers. Lithospermic acid presented an $[M-H]^-$ at $m/z = 537$ amu, which fragmented to 493 and 359 amu, in accordance with the mass spectral data reported by Martins et al. (2014),

Miron et al. (2013) and Liu et al. (2007). Also, the UV–vis data of the above researchers are close to the present research. The peak attributed to salvianolic acid A presented $[M-H]^-$ at $m/z = 493$ amu, which fragmented to 295 amu. Liu et al. (2007) reported the same results for salvianolic acid A. Moreover, Xu et al. (2008) and Zhang et al. (2013) presented the UV–vis spectra of salvianolic acid A and the pattern of each spectrum appears identical to ours. The elution order observed in the current research, i.e. rosmarinic, followed by lithospermic and salvianolic acid A, was verified by the results of Liu et al. (2007). Additionally, caffeic acid was detected in minor quantities in EtOH and Aq, as well as an unidentified phenolic acid that presented a UV spectrum similar to rosmarinic acid.

Ethanol recovered the highest quantities of phenolic acids, while appreciable amounts were detected in the Aq extract (Table 1). Overall, the content of phenolic acids was higher than that of flavonoids in Aq and EtOH, contrary to EAc.

The monophenol carvacrol that was one of the main constituents of the EO, was not totally recovered by water-steam distillation, and a small but appreciable amount was quantified in EAc, while it was also detected in Aq.

The TP content, measured by the Folin Ciocalteau method, is also presented in Table 1, and followed the order EtOH > Aq = EO > EAc.

3.3. Antiradical activity of the EO and extracts

The results of the EO and extracts against DPPH radical are presented in Table 1. EtOH and Aq exhibited a good antiradical activity, EAc a moderate one, while EO a very low one. The fact is explained by the low antiradical activity of the main phenolic component of EO, namely carvacrol. The EC_{50} value of carvacrol was determined equal to 717 g/kg DPPH in the current research, while γ-terpinene, the main non-phenolic component of EO, was almost inactive (EC_{50} = 143000 g/kg DPPH).The EC_{50} values of the extracts (EAc, EtOH, and Aq) were correlated to the TP content ($r = -0.98$), as well as total phenolic acids concentration ($r = -0.83$). This fact indicates that, in addition to the phenolic acids that demonstrate a high activity against DPPH radical, the flavonoid compounds of the extracts contributed to antiradical activity. Rosmarinic acid followed by lithospermic and salvianolic acid are main constituents of the extracts (Tables 1 and 2). The ratio of total phenolic acids/TP ranges between 0.3 in EAc and 0.7 in EtOH. The three identified phenolic acids are potent radical scavengers due to their catecholic units. Rosmarinic acid presents two catecholic units and the EC_{50} value of the pure compound was determined equal to 41 g/kg DPPH in the current research. Lithospermic and salvianolic acid are expected to be equal and more active, since they bear two and three catecholic units respectively.

3.4. Antioxidant activity in bulk palm oil and oil-in-water emulsions

Fig. 2 presents the results of the addition of EAc to palm oil oxidation. EtOH and Aq could not be properly diluted in oil, due to their content in polar compounds, therefore they were not further examined. Both enriched and non-enriched samples presented a quasi linear increase of PV versus time, after a short induction period. EAc prolonged slightly the induction period, while decreased by 42% the rate of peroxide accumulation (i.e. 1.92 d^{-1}, as compared to 3.30 d^{-1} for the control sample). This result indicates that the phenolic compounds present in the EAc of *S. thymbra* are capable of protecting palm oil against oxidation and prolong its shelf life.

The experimental data for primary and secondary oxidation products of the emulsions are shown in Fig. 3. EtOH and Aq depressed the formation of PV, while EAc demonstrated very low activity (Fig. 3a). Similar results were obtained for conjugated dienes (data not presented). EAc contains mainly nonpolar compounds that are soluble in the oil. Thus, they are probably located inside the oil droplets and not at the interface, where the free radicals are formed. Hence, the phenolic components of the extract cannot protect the oil from oxidation. On the contrary, EtOH containing more polar compounds was very effective in slowing down the primary oxidation process.

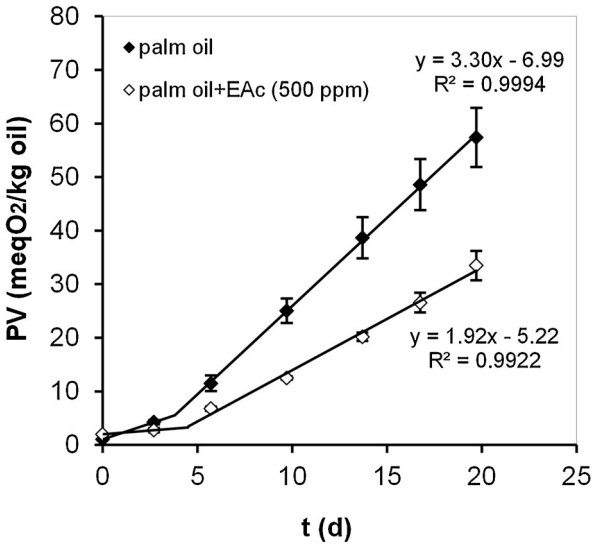

Fig. 2. The effect of ethyl acetate extract (EAc) on the primary oxidation process of palm oil at 70 °C monitored with Peroxide Value (PV).

The primary oxidation products tended to decrease at the end of the oxidation period probably because they degraded to secondary oxidation products, as shown in Fig. 3b. In fact, the secondary oxidation products of all samples, as estimated by *p*-AV, remained low throughout the oxidation and tended to increase at the end, except the emulsion enriched with Aq. Consequently, the low peroxide accumulation in the sample with Aq, which is observed in Fig. 3a, should be rather attributed to their decomposition to secondary oxidation products instead of lower formation rate induced by the additive. Aq contains copper ions from the distiller (6 ppm) that accelerate the oxidation and therefore is not an appropriate additive to prolong the shelf life of lipid foods.

The overall assessment of the protection capacity of the three extracts can be done through the Totox value = (2xPV + p-AV) shown in Fig. 3c. It is obvious that the EtOH, rich in phenolic acids and flavonoid glycosides, offers the best protection against emulsion oxidation.

3.5. Antimicrobial activity

3.5.1. Antimicrobial activity of the EO and extracts

The antimicrobial activity of *S. thymbra* EO and its extracts (Aq, EAc and EtOH) was evaluated against seven common food spoilage and pathogenic bacteria (Burt, 2004; Ercolini et al., 2007; Lianou and Koutsoumanis, 2013), as well as against *S. cerevisiae* and *A. niger*, which have been previously used as model systems in food spoilage (Battey et al., 2002; Garcia et al., 2009). The results of the disc diffusion method are presented in Table 3, while the MIC and NIC values in Table 4.

The data obtained from the disc diffusion method indicated that all microbes tested were sensitive to *S. thymbra* EO (Table 3), while EAc and EtOH were active only against the bacteria species, but not against *S. cerevisiae* and *A. niger*. Of note, inhibition zones for *A. niger* were 45 ± 0.7, 30 ± 0.7 and 13 ± 1.0 mm for the first 3 days respectively; the inhibition zones disappeared during incubation for longer time periods (data not shown). In general, inhibition

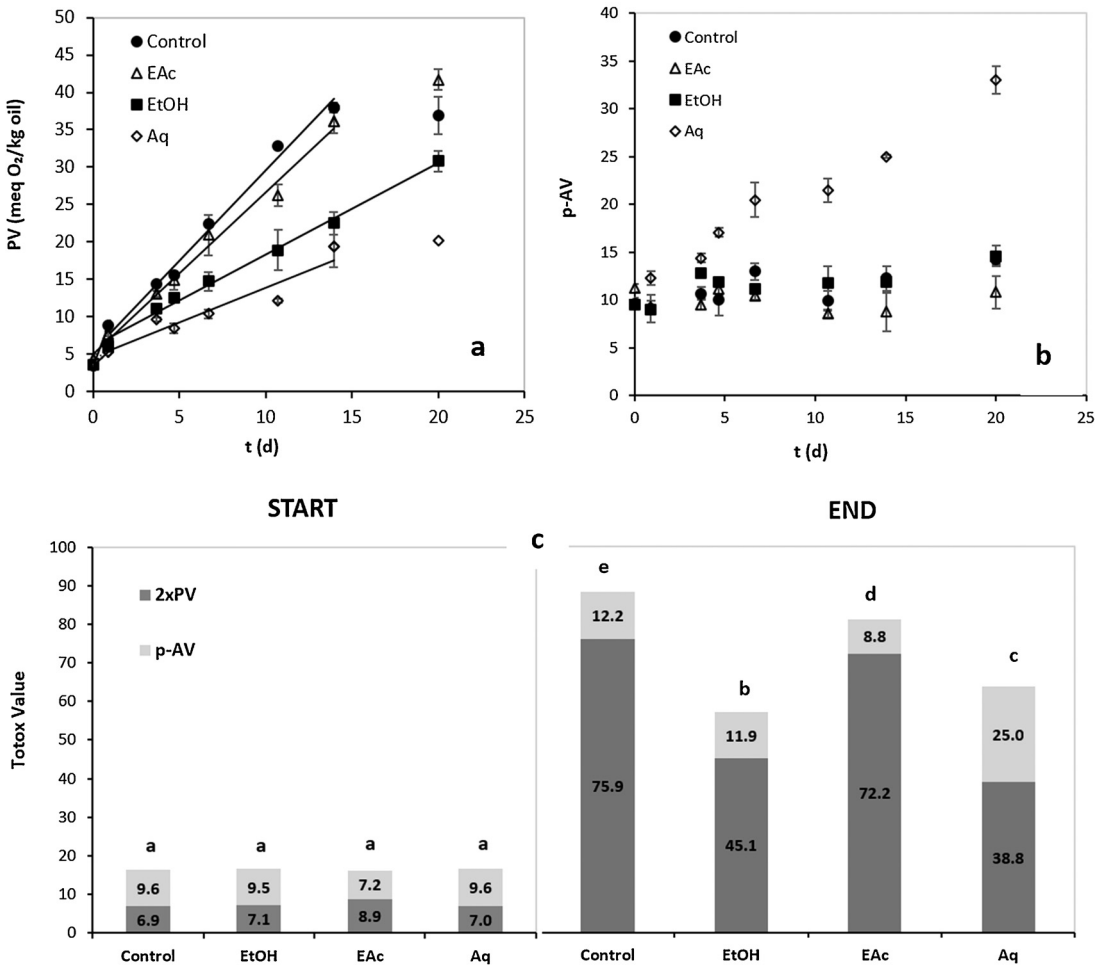

Fig. 3. The effect of ethyl acetate (EAc), ethanol (EtOH) and water extract (Aq) of *S. thymbra* on slowing down (a) the peroxide value (PV), (b) the *p*-anisidine value (*p*-AV) during the oxidation of palm oil-in-water emulsions 30% (w/w), at 70 °C (a), and (c) the resulting totox value at the beginning and the end of oxidation.

Table 3
Antimicrobial activity of *Satureja thymbra* essential oil (EO), ethanol (EtOH), and ethyl acetate (EAc) extracts against common spoilage and pathogenic microorganisms monitored by the disk diffusion assay. The inoculums tested were 5 and 7 logcfu/mL for bacteria and *S. cerevisiae*. The diameter (mm) of the inhibition zone is the mean of six observations from three independent experiments.

Microbial species	EO		EtOH		EAc	
	Initial inoculum (logcfu/mL)					
	5	7	5	7	5	7
S. Enteritidis	28 ± 0.6	20 ± 0.6	15 ± 0.7	12 ± 0.7	10 ± 0.6	8 ± 0.7
S. Typhimurium	30 ± 0.6	25 ± 0.6	13 ± 0.7	11 ± 0.7	10 ± 0.7	8 ± 0.6
E. coli	32 ± 0.6	23 ± 0.6	13 ± 0.6	12 ± 0.6	10 ± 0.7	9 ± 0.7
L. monocytogenes	35 ± 0.6	30 ± 0.6	13 ± 0.7	10 ± 0.7	10 ± 0.7	8 ± 0.7
S. epidermidis	35 ± 0.6	25 ± 0.6	13 ± 0.7	11 ± 0.6	12 ± 0.6	11 ± 0.7
S. aureus	25 ± 0.6	20 ± 0.6	12 ± 0.7	10 ± 0.7	10 ± 0.7	9 ± 0.7
P. fragi	21 ± 0.5	9 ± 0.5	11 ± 0.5	8 ± 0.7	13 ± 0.5	7 ± 0.5
S. cerevisiae	50 ± 0.5	40 ± 0.7	0	0	0	0

zones were significantly larger for the EO, followed by EtOH and EAc. Specifically, the highest inhibition zone was recorded for *S. thymbra* EO in *S. cerevisiae* (50 mm) and the lowest for EAc in *P. fragi* (7 mm). On the contrary, the Aq showed no antimicrobial activity (data not shown).

Although the inhibition zone method is widely used for the evaluation of the antimicrobial activity of essential oils, there are various factors that influence the outcome of the results, such as the inoculum size, the ability of the essential oil to diffuse uniformly through the agar, etc (Chorianopoulos et al., 2004; Soković et al., 2010).

Table 4
MIC and NIC (g dry extract or EO/L) of *Satureja thymbra* essential oil (EO), ethanol (EtOH), and ethyl acetate (EAc) extracts against common spoilage and pathogenic bacteria. MIC and NIC standard deviation for EtOH and EAc ranged <0.001.

Microbial species	*Satureja thymbra*					
	EO		EtOH		EAc	
	MIC	NIC	MIC	NIC	MIC	NIC
S. Enteritidis	1.07 ± 0.009	0.64 ± 0.009	0.19	0.08	0.46	0.34
S. Typhimurium	0.73 ± 0.018	0.27 ± 0.009	0.19	0.07	0.44	0.32
E. coli	1.16 ± 0.018	0.55 ± 0.009	0.19	0.08	0.47	0.37
L. monocytogenes	1.39 ± 0.009	0.07 ± 0.009	0.20	0.08	0.46	0.29
S. epidermidis	1.71 ± 0.018	1.02 ± 0.018	0.20	0.09	0.40	0.27
S. aureus	1.11 ± 0.009	0.55 ± 0.009	0.19	0.06	0.55	0.44
P. fragi	3.20 ± 0.018	0.69 ± 0.009	0.23	0.10	0.48	0.28

MIC and NIC determination documented the effective growth inhibition of *S. thymbra* EO and extracts against all bacteria tested (Table 4). MIC and NIC values for the EO were higher compared to EtOH and EAc. Hence, the lowest MIC and NIC values for *S. thymbra* EO were observed in *S.* Typhimurium (0.73 g/L) and *L. monocytogenes* (0.07 g/L), respectively, while the corresponding highest values for EtOH were noted in *P. fragi* (0.23 and 0.10 g/L, respectively) and for EAc in *S. aureus* (0.55 and 0.44 g/L). Noticeably, to the best of our knowledge, this is the first report concerning the antimicrobial activity of *S. thymbra* extracts (EAc and EtOH) obtained from

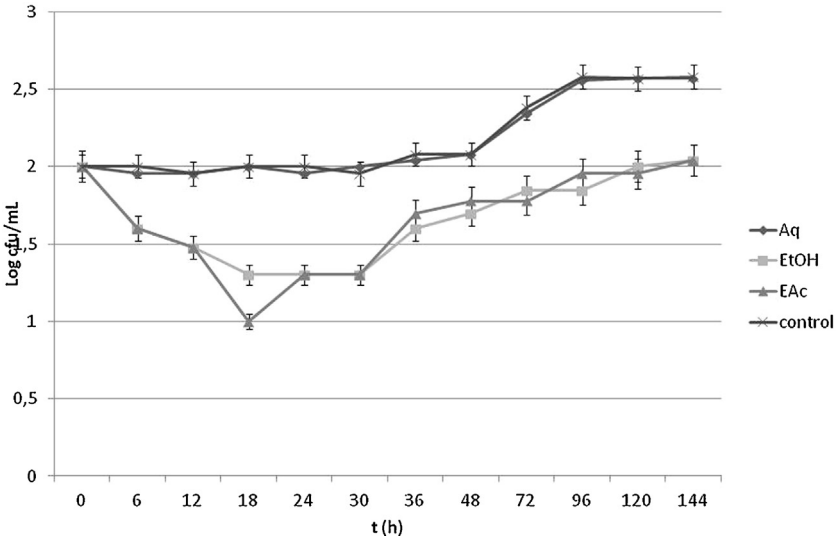

Fig. 4. Counts of *L. monocytogenes* in emulsions containing *S. thymbra* extracts stored at 4 °C after deliberate inoculation (initial inoculum 10^2 cfu/mL). Aq: water extract, EtOH: ethanol extract, EAc: ethyl acetate extract, control: emulsion with no extract.

the residue of distillation. The antimicrobial activity of *S. thymbra* EO and its extracts could be attributed to the action of their main constituents, mainly carvacrol, γ-terpinene, *p*-cymene, lithospermic and rosmarininic acids, as well as other phenolic compounds and flavonoids (Cushnie and Lamb, 2005; Giweli et al., 2012; Zhao et al., 2011; Abedini et al., 2013; Mitropoulou et al., 2015), although possible synergistic/antagonistic effects should not be excluded (Burt, 2004; Nazzaro et al., 2013; Bassolé and Juliani, 2012). Such effects must be further studied using food model systems consisting of various mixtures of pure compounds.

Similar results about high antimicrobial activity of EOs derived by various *Satureja* species rich in carvacrol were previously published (Chorianopoulos et al., 2004, 2006a,b; Serrano et al., 2011). Variations in the chemical composition of *Satureja* EOs are reported, as it may be influenced by many factors, such as environmental conditions, climate and time of harvesting (Chorianopoulos et al., 2006b). Chorianopoulos et al. (2006a) investigated the effect of the harvesting time on the antimicrobial activity of *S. thymbra* and *S. parnassica* EOs reporting similar MIC and NIC values to our research, but their study was limited to only two microbial species (*S.* Enteritidis and *L. monocytogenes*). On the other hand, the antimicrobial potential of *S. montana* and *S. spinosa* EOs were previously assessed (Serrano et al., 2011; Chorianopoulos et al., 2006b). Both studies concluded that the EOs isolated by *Satureja* species have strong potential for use as natural antimicrobials in the preservation of processed foods. Although similar MIC and NIC values were reported by Chorianopoulos et al. (2006b) for *S. spinosa* EO, MIC values calculated by Serrano et al. (2011) for *S. montana* EO ranged in 0.80–2.10 μg/mL and 0.04–30.30 mg/L, respectively. Noticeably, determination of MIC and NIC values by most of the above studies was based on assays with limited accuracy and not on an efficient, precise and sophisticated methodology (Lambert and Lambert, 2003). Apparently, precise determination of MIC and NIC values is crucial for the food industry, in order to regulate the optimum amount of the antimicrobial agent to secure microbial safety. Additionally, to the best of our knowledge, this is the first study reporting considerable antibacterial activity of post distillation by-products of *S. thymbra*.

3.5.2. Antimicrobial activity in oil-in-water emulsions

The antimicrobial activity of *S. thymbra* extracts was further evaluated in oil-in-water emulsions that were deliberately spiked with *L. monocytogenes*. An experimental inoculation with 100 cfu/mL was performed, as this is the upper limit for *L. monocytogenes* viable counts in foods, according to EU regulations (European Commision regulation No. 2073/2005). The viable counts were monitored during storage at 4 °C for up to 144 h and the results are presented in Fig. 4. Levels of *L. monocytogenes* dropped immediately after inoculation in emulsions containing EtOH or EAc and remained in significantly ($p < 0.05$) lower levels compared to the control sample and to emulsion containing the Aq. Consequently both EtOH and EAc can protect emulsions against food pathogens, while EtOH combines both antimicrobial and antioxidant properties (Fig. 3).

4. Conclusions

The extracts obtained from the residue remaining after the distillation of *Satureja thymbra* are rich in phenolic acids and flavonoid compounds. The results of the present work revealed that the EAc and EtOH depressed the oxidation of palm oil and palm oil-in water emulsions, respectively. Moreover, the EO, as well as the EtOH and EAc are noteworthy growth inhibitors of food spoilage and pathogenic microbes, indicating that they represent effective and inexpensive sources of potent natural antimicrobial and antioxidant agents, which may be incorporated in food products to extent shelf life. Overall, the results of the present work indicated that in addition to the EO, the by-product generated by the distillation of the herb can be exploited for the recovery of extracts rich in bioactive compounds. However, further research to elucidate their exact mode of action and to determine the safety of the long-term use of high doses, is required in order to expand the use of *S. thymbra* extracts in industrial practice and food technology.

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