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Antioxidant Activities and Phenolic Composition of Extracts from Greek Oregano, Greek Sage, and Summer Savory

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Oregano vulgare L. ssp. *hirtum* (Greek oregano), *Salvia fruticosa* (Greek sage), and *Satureja hortensis* (summer savory) were examined as potential sources of phenolic antioxidant compounds. The antioxidant capacities (antiradical activity by DPPH• test, phosphatidylcholine liposome oxidation, Rancimat test) and total phenol content were determined in the ethanol and acetone extracts of the dried material obtained from the botanically characterized plants. The ground material was also tested by the Rancimat test for its effect on the stability of sunflower oil. The data indicated that ground material and both ethanol and acetone extracts had antioxidant activity. Chromatographic (TLC, RP-HPLC) and NMR procedures were employed to cross-validate the presence of antioxidants in ethanol and acetone extracts. The major component of all ethanol extracts was rosmarinic acid as determined by RP-HPLC and NMR. Chromatography indicated the presence of other phenolic antioxidants, mainly found in the acetone extracts. The presence of the flavones luteolin and apigenin and the flavonol quercetin was confirmed in the majority of the extracts by the use of a novel ^{1}H NMR procedure, which is based on the strongly deshielded OH protons in the region of 12−13 ppm without previous chromatographic separation. This deshielding may be attributed to the strong intramolecular six-membered ring hydrogen bond of the OH(5)···CO(4) moiety.

KEYWORDS: Phenolic antioxidants; Greek oregano; Greek sage; summer savory

INTRODUCTION

Aromatic plants have been studied as sources of different classes of natural antioxidants. Some plants, grown wild or cultivated, have been exploited commercially for many years (*1*−*4*). Among the various medicinal and culinary herbs, some endemic species are of particular interest for small countries because they may be used for the production of raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits.

A number of plants characterized by a high carvacrol content, known as "oregano" plants (*5*, *6*), and *Satureja hortensis* (summer savory), a closely related plant (*7*), have been reported as sources of rosmarinic acid and other phenols (*8*, *9*), but their compositional data are incomplete and they are not exploited to the same extent as *Rosmarinus officinalis*. *Salvia fruticosa*, known as Greek sage, that may also be rich in rosmarinic acid (*10*, *11*), has not received much attention in comparison to *Salvia officinalis* (*12*−*14*).

The purpose of this study was to evaluate three perennial plants of the family Lamiaceae, *Oregano vulgare* L. ssp. *hirtum* (Link) Ietswaart (Greek oregano) and *S. fruticosa* (Greek sage), collected in Greece, and *S. hortensis* (summer savory), collected in Lithuania. The plants were examined as potential sources of phenolic antioxidant compounds within the framework of research projects for the development of preparations containing natural antioxidants. Commonly used assays (DPPH radical scavenging, liposome and Rancimat tests) and chromatographic (TLC, RP-HPLC) and NMR procedures were employed to cross-validate the importance of the plant material to provide extracts containing potent antioxidants.

MATERIALS AND METHODS

Plant Material. Dried material from botanically characterized *O. vulgare* ssp. *hirtum* and *S. fruticosa* was a gift of the Botany Department (Mediterranean Agronomic Institute of Chania, Crete, Greece). Lithuanian *S. hortensis* was collected, characterized, and dried under the guidance of Professor R. Venskoutonis (Kaunas University, Vilnius, Lithouania). The summer savory of Bulgarian origin was a commercial sample. The dried material was ground to pass a 0.4 mm sieve and

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extracted with ethanol and acetone in a Soxhlet apparatus for 6 h. The extracts were purged with nitrogen and kept in a dark and cool place until analyzed. The percent yield of essential oil was measured in a Clevenger apparatus. Plant material was collected immediately after blooming and air-dried at room temperature in the dark.

Standards. Caffeic acid (97%) and rosmarinic acid (97%) were from Fluka AG (Buchs, Switzerland). Chlorogenic acid (97%), quercetin (99%), and rutin (90%) were from Riedel de Haën (Seelze, Germany). Apigenin (95%) and kaempferol were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Luteolin (95%) was from Röth (Karlsruhe, Germany).

Solvents and Reagents. All solvents and reagents from various suppliers were of the highest purity needed for each application. The Folin−Ciocalteu reagent was from Merck (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH•), L-α-phosphatidylcholine (lecithin, ∼40%) from soybean, and copper acetate monohydrate were purchased from Sigma (St. Louis, MO).

Apparatus. A U-2000 Hitachi spectrophotometer (Tokyo, Japan) was used for the measurement of absorbance in the UV/vis region. Induction periods of lipid substrates were measured using a Rancimat 617 apparatus (Metrohm AG, Herisau, Switzerland). A high-performance liquid chromatograph consisting of a Thermoquest Spectra System quaternary pump, model P4000 (Austin, TX), a Rheodyne Model 9725 (Cotati, CA) injection valve with a 20 μL fixed loop, a Laballiance column oven, model 505 (State College, PA), and a diode array Fasma 406 scanning detector (HPLC/DAD) (Rigas Laboratories, Thessaloniki, Greece) was used. The analysis of phenolic compounds was performed on a 125 mm × 4 mm i.d., 5 μm, Nucleosil 100-5 C_{18} column (Macherey-Nagel, Düren, Germany). The data from the diode array detector were stored and processed with EZChrom chromatographic software (Scientific Software Inc., San Ramon, CA). All NMR experiments were performed on a Bruker AMX-400 MHz spectrometer equipped with a *z*-gradient unit. Spectra were obtained from ethanol and acetone extracts dissolved in CD_3OH and CD_3COCD_3, respectively. Chemical shifts and integrals were measured with reference to the internal standard TMSP-d_4 or TMS ($\delta = 0.000$), of known concentration, depending on the solvent used. NMR data were processed using UXNMR (Bruker) software. 2D NMR parameters are described in previous studies (*15*).

Determination of Total Phenol Content of Plant Extracts. The content of total phenols in the plant extracts was measured by the Folin−Ciocalteu assay (*16*). Results were expressed as mg of caffeic acid/100 g of extract. Yields of the extracts were based on weight difference.

Estimation of Antiradical Activity by the DPPH• Test. The antiradical activity of extracts, containing 50 mg/100 g of total phenols expressed as caffeic acid, was determined according to ref *17*. Results were expressed as DPPH• % scavenging $= a \times 100/b$, where $a = [(abs_{initial} - abs_t)/abs_{initial}]_{extract}$ and $b = [(abs_{initial} - abs_t)/abs_{initia)}]_{caffeic\ acid}$. Caffeic acid solution (500 mg/L of ethanol) was conventionally accepted to result in 100% reduction of the DPPH radical.

Rancimat Test. Samples of sunflower oil (2.5 g) containing 0.02% w/w extract or 2% w/w ground material were subjected to oxidation at 120 °C (air flow 20 L/h). Induction periods, IP (h), were recorded automatically in duplicate. The coefficient of variation (CV) (%) of the method was 3.3 ($n = 3$). Protection factors (PF) were then calculated from these values ($PF = IP_{extract}/IP_{control}$).

Phosphatidylcholine Liposome Oxidation. Lecithin was suspended in doubly distilled water at a concentration of 8 mg/mL by stirring with a glass rod and sonication for approximately 5 min. Liposome formation was obtained through additional sonication with a rod (UP 200S, Dr. Hielscher, GmbH, Berlin, Germany) (2.5 min for 10 mL aliquots of the liposome sample). Ethanol solutions of the plant extracts were added into Erlenmeyer flasks at a final concentration of approximately 50 mg/100 g. Caffeic acid solutions (500 mg/L) were used for comparison. Liposome aliquots were weighed into the flasks and diluted with doubly distilled water to a final lecithin concentration of 0.8% w/w. The samples were oxidized by adding cupric acetate (3 μM) and shaking at 37 °C in the dark. Liposome oxidation was monitored according to ref *18*.

Table 1. Essential Oil Content, Extraction Yield, Total Phenol Content (mg of Caffeic Acid/100 g of Extract), and Antioxidant Activity of Ethanol Extracts of the Plant Species under Investigation

plant species	essential oil (% plant material)[a]	% yield of extract	total phenol content[b]	DPPH• (% scavenging)[c]
O. vulgare ssp. *hirtum*[d]	3.7	46.5	9800	30.1 ± 0.9
O. vulgare ssp. *hirtum*[e]	6.1	45.5	15100	34.8 ± 1.5
S. hortensis[e,f]	0.2	66.9	13200	75.2 ± 0.6
S. hortensis[g]	2.6	30.3	11600	49.9 ± 0.4
S. fruticosa[e]	1.1	82.6	5000	33.2 ± 1.2

[a] As determined in a Clevenger apparatus. [b] Mean value of two measurements. [c] Mean value of three measurements ± SD (standard deviation). [d] Grown wild. [e] Cultivated. [f] Lithuanian origin. [g] Bulgarian origin.

Chromatographic Analyses. Thin-layer chromatography was conducted on analytical silica gel plates (Riedel de Haën, Seelze, Germany). The presence of specific phenolic compounds was detected by comparison with standards of caffeic, chlorogenic, and rosmarinic acids, quercetin, rutin, apigenin, kaempferol, and luteolin. Two different developing systems were used: (a) $CHCl_3/CH_3COOEt/HCOOH$, 5:4:1 (v/v/v) (*19*), and (b) $CH_3COOEt/CH_3OH/H_2O$, 77:13:10 (v/v/v) (*20*). For visualization, plates were sprayed with (a) $FeCl_3$ (2% in ethanol) and (b) $AlCl_3$ (1% in ethanol) (*21*). For the HPLC/DAD analysis of the selected extracts, 1% v/v formic acid (solvent A) and acetonitrile (solvent B) were used (*22*). The elution system was as follows: 0−10 min, 10−13% solvent B; 10−25 min, 13−70% solvent B; 25−29 min, 70% solvent B; 29−30 min, 70−10% solvent B; 30−40 min, 10% solvent B. The flow rate was 1 mL/min, and the injection volume was 20 μL. Peak characterization was based on spiking with standards and UV spectra matching. Eight point calibration curves, constructed for rosmarinic acid and other standards, were used for quantification.

Nuclear Magnetic Resonance Spectroscopy. NMR spectra of the ethanol and acetone extracts were obtained in CD_3OH and CD_3COCD_3 solutions without prior chromatographic separation. For the differentiation of caffeic from rosmarinic acid, a combination of variable-temperature 2D NMR techniques was used (*15*). The methodology was a combination of variable-temperature two-dimensional $^1H-^1H$ double-quantum-filtered correlation spectroscopy ($^1H-^1H$ DQF-COSY), $^1H-^{13}C$ heteronuclear multiple-quantum coherence ($^1H-^{13}C$ HMQC), and $^1H-^{13}C$ heteronuclear multiple-bond correlation ($^1H-^{13}C$ HMBC) gradient NMR spectroscopy. For the identification of apigenin, luteolin, and quercetin, only conventional 1D proton NMR spectroscopy was used. The suppression of the water resonance was achieved with the use of the Watergate pulse sequence for gradient (*23*).

RESULTS AND DISCUSSION

A screening assay of the antioxidant activity of ethanol extracts from the three plants indicated that all of them have a high capacity to scavenge free radicals (**Table 1**). This capacity coincides with a high total phenol content, but it is not proportional. Thin-layer chromatography verified the presence of phenolic compounds; the main component appeared to be rosmarinic acid while three different bands of flavonoids were also detected.

In light of this evidence, new plant material was selected just after blooming for further examination. A screening of the antioxidant activity of the dried material using the Rancimat test indicated that all of the plants were good sources of antioxidants: $PF_{O.vulgare}$, 2.1; $PF_{S.hortensis}$, 1.3; and $PF_{S.fruticosa}$, 3.4. Ethanol and acetone extracts were then prepared. Ethanol was chosen as an industrial polar nontoxic solvent and acetone as a less polar solvent, often used to extract active phenolic constituents from plants (*2*, *24*, *25*). The total phenolic content

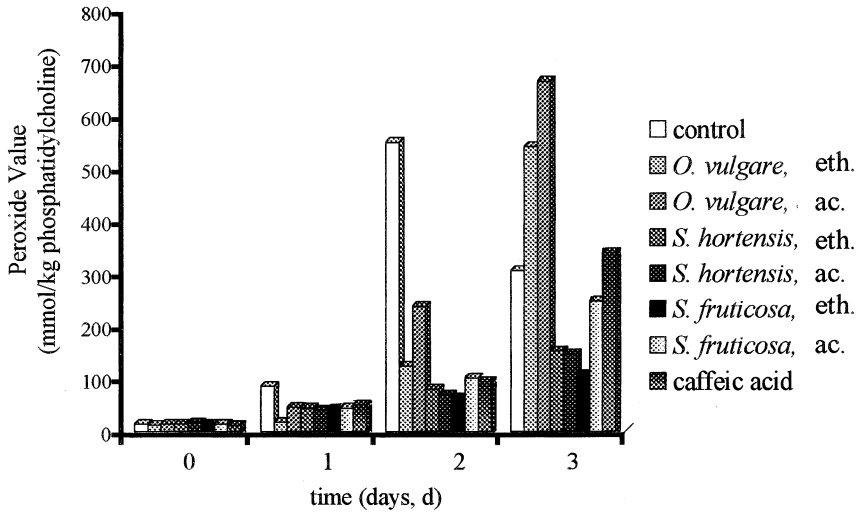


Figure 1. Antioxidant activity of ethanol and acetone extracts (50 mg of caffeic acid/100 g) in lecithin liposomes at 37 °C.

Table 2. Essential Oil Content, Extraction Yield, Total Phenolic Content (mg of Caffeic Acid/100 g of Extract), and Antioxidant Activity of Ethanol and Acetone Extracts of the Plants under Investigation

plant extract	essential oil (% plant material)[a]	% yield of extract	total phenol content[a]	DPPH• (% scavenging)[b]	PF[b,c]
O. vulgare ssp. *hirtum*	2.9				
ethanol		59.2	9700	99.1	1.2
acetone		22.9	17400	54.4	1.4
S. hortensis	0.7				
ethanol		31.4	6400	95.8	1.2
acetone		9.9	21500	33.0	1.2
S. fruticosa	0.9				
ethanol		61.9	8100	98.5	1.7
acetone		23.8	9900	97.6	2.2

[a] As determined in a Clevenger apparatus. [b] Mean value of two measurements; CV (%) = 3, $n = 5$. [c] PF = protection factor.

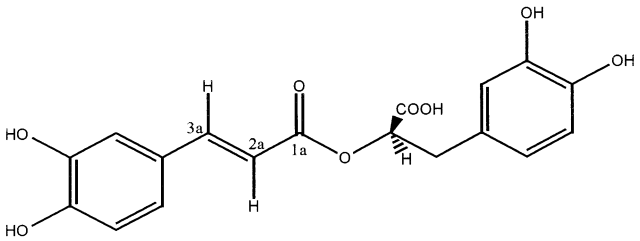
Table 3. Caffeic and Rosmarinic Acid Content of Ethanol and Acetone Extracts As Determined by RP-HPLC at 326 nm and NMR Techniques

	caffeic acid (mg/100 g of plant extract)		rosmarinic acid (mg/100 g of plant extract)	
plant extract	HPLC	NMR	HPLC[a]	NMR[b]
O. vulgare ssp. *hirtum*				
ethanol	tr[c]	tr	1271 ± 115	904 ± 74
acetone	nd[d]	nd	231 ± 15	173 ± 12
S. hortensis				
ethanol	tr	tr	2137 ± 223	2517 ± 215
acetone	nd	nd	249 ± 11	267 ± 17
S. fruticosa				
ethanol	nd	nd	1483 ± 154	1185 ± 83
acetone	nd	nd	556 ± 53	359 ± 29

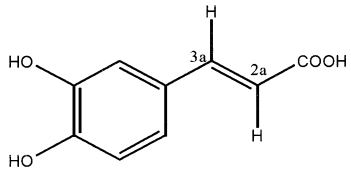
[a] Mean value of three measurements ± SD (standard deviation). [b] Mean value of four measurements ± SD (standard deviation). [c] tr = traces. [d] nd = not detected.

of the new extracts was measured, and their antioxidant activity was examined using three different assays. Extracts were tested toward their DPPH• scavenging activity. Their efficiency toward oxidation of bulk oil under accelerated conditions (120 °C) was evaluated using the Rancimat apparatus. Finally, the activity of the extracts in a multiphase system was examined using the phosphatidylcholine liposome assay (*18*). Results of the DPPH• and Rancimat tests and total phenol content of the extracts are presented in **Table 2**. Results from the phosphatidylcholine liposome assay are given in **Figure 1**. The data indicated that the six extracts had antioxidant activity. Differences were observed in the activity between the ethanol and acetone extracts, which cannot be explained by the total phenol content. This can be attributed to the different composition of the ethanol and acetone extracts. The active compounds of the essential oils of *O. vulgare* and *S. hortensis* (mainly carvacrol) may contribute to the antioxidant activity (*26*). This effect is not shown in the Rancimat test because of the volatility of these phenols. The essential oil of *S. fruticosa* does not contain carvacrol or thymol or other antioxidants in the volatile fraction, and thus the difference in the polarity of the solvent does not affect the scavenging activity (*11*). Greek oregano, summer savory, and Greek sage seem to be good sources of natural antioxidants and can be further considered for commercial exploitation.

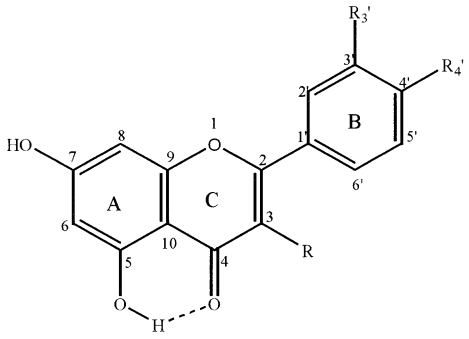
Assignment of the antioxidant activity to specific phenolic compounds was attempted using chromatographic and NMR procedures. Thin-layer chromatography indicated the presence of rosmarinic acid in both ethanol and acetone extracts. Caffeic acid seemed to be present only in the ethanol extracts of *O. vulgare* ssp. *hirtum* and *S. hortensis.* Bands of flavonoids were also observed near the baseline and the solvent front on the TLC plates. RP-HPLC was used to characterize further the phenolics of each extract and to check the levels of caffeic and rosmarinic acids. The respective HPLC data are shown in **Table 3**. High levels of rosmarinic acid were found in all extracts whereas the presence of caffeic acid in trace amounts was confirmed in the extracts of oregano and summer savory. Comparing the results of **Tables 2** and **3**, it can be generally concluded that the extracts that are rich in rosmarinic acid have higher radical scavenging activity. This caffeic acid derivative, common in many plants and very often present in our diet, is a strong radical scavenger and has been reported to be more effective in relation to Trolox (*27*). The presence and levels of caffeic and rosmarinic acids in the extracts were also examined by the use of two-dimensional NMR spectroscopy (*15*) without previous chromatographic separation. To improve resolution of strongly overlapped signals, techniques based on the temperature dependence of proton chemical shifts were applied (*15*). In practice, by variable-temperature 2D DQF-COSY spectra at 243 K, the cross-peak connectivity of the H_{3a} and H_{2a} protons of rosmarinic acid (**Figure 2**) is differentiated from that of caffeic acid (**Figure 2**) and other overlapping cross-peaks. Further



Rosmarinic acid (**1**)



Caffeic acid (**2**)



Apigenin: R_3=H, $R_{3'}$= H, $R_{4'}$=OH (**3**)
Luteolin: R_3=H, $R_{3'}$= OH, $R_{4'}$=OH (**4**)
Quercetin: R_3=OH, $R_{3'}$= OH, $R_{4'}$=OH (**5**)

Figure 2. Structural formulas of rosmarinic acid (**1**), caffeic acid (**2**), and apigenin (**3**), luteolin (**4**), and quercetin (**5**).

improvement in resolution and better assignment information were achieved by the use of two-dimensional $^1H-^{13}C$ heteronuclear techniques. The NMR analysis confirmed the presence of rosmarinic and caffeic acids and cross-validated the levels obtained by HPLC (**Table 3**).

The antioxidant activity of the extracts may also be partly due to the presence of other phenolic compounds such as flavonoids. On the HPLC chromatograms some peaks having the same RRTs with apigenin and luteolin or quercetin (**Figure 2**) were located. These peaks had UV spectra that practically matched fully with those of the respective standards. The literature related to the presence of such flavonoids in the plants under investigation is generally limited. Apigenin and quercetin derivatives have been found in oregano plants (*28*, *29*). Karakaya et al. (*30*) reported the presence of quercetin and luteolin in *S. officinalis* infusions. The flavonoid composition of *S. fruticosa* and *S. hortensis* is not known.

The presence of these three flavonoids was investigated using NMR techniques. The ^1H NMR spectra of the three flavonoids in CD_3COCD_3 and CD_3OH solutions indicated a significantly deshielded signal in the region of 12−13 ppm, as shown in **Figure 3**. This resonance may be attributed to the hydroxyl proton OH(5) of flavonoids which participates in a strong six-membered ring intramolecular hydrogen bond with CO(4) and, therefore, is strongly deshielded (*31*) (**Figure 2**). As a rule the ^1H NMR resonances of the −OH groups appear at room temperature as broad signals especially in protic solvents, owing

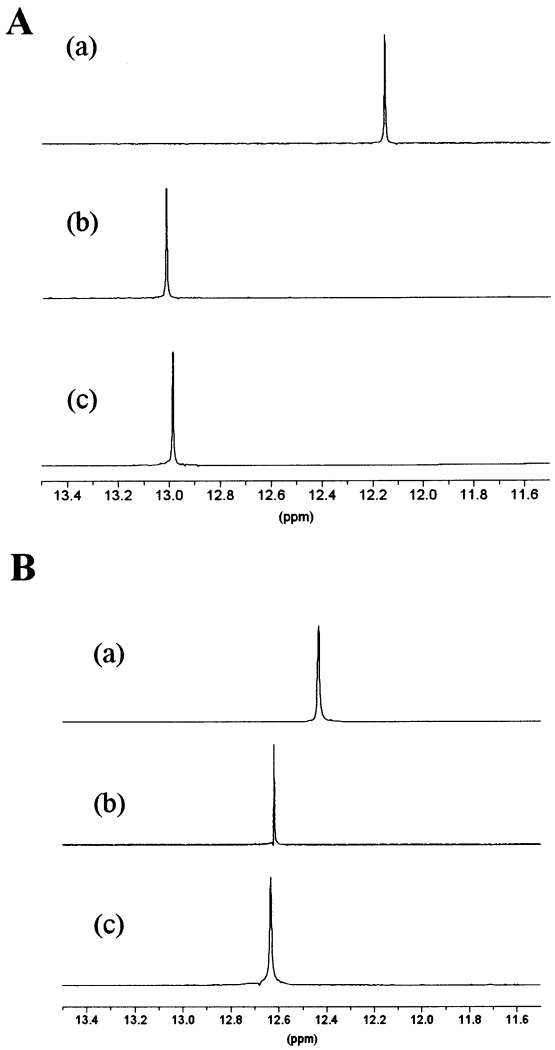


Figure 3. Selected region of the 400 MHz ^1H NMR spectra of (**A**) (a) quercetin, (b) luteolin, and (c) apigenin, in acetone-d_6 at 300 K, and of (**B**) (a) quercetin, (b) luteolin, and (c) apigenin, in methanol-d_3 at 240 K.

to the mobility of the hydrogen and its fast exchange, on the NMR time scale, with the protons of the solvent. However, by decreasing the temperature, the proton exchange rate is reduced and the −OH peaks are revealed as sharp peaks (*32*). In acetone solution the OH(5) resonances of the three flavonoids are clearly observed as sharp singlets even at room temperature (**Figure 3A**). In methanol-d_3 solution a relatively broad −OH(5) resonance at 12−13 ppm commences to appear at 280 K. Variable-temperature ^1H NMR spectroscopy of apigenin, luteolin, and quercetin in CD_3OH showed that at 240 K the OH(5) signals appeared as sharp singlets (**Figure 3B**). The observation of the OH signal in protic solvent was achieved by the use of the Watergate pulse sequence for gradient, which does not eliminate fast-exchanging OH resonances with the solvent. Since the region of 12−13 ppm in the ^1H NMR spectra of the extracts is not as crowded as the aromatic one, the identification of flavonoids can be based only on 1D proton NMR spectroscopy, without the need for two-dimensional techniques, which are very time-consuming. It is notable that the OH(5) resonance is more deshielded in the flavones apigenin and luteolin compared to that of the flavonol quercetin. This can be attributed to the presence of the OH(3) group in quercetin (**Figure 2**) that attenuates the electron density of the C(O) oxygen and, thus, decreases the strength of the OH(5)···OC

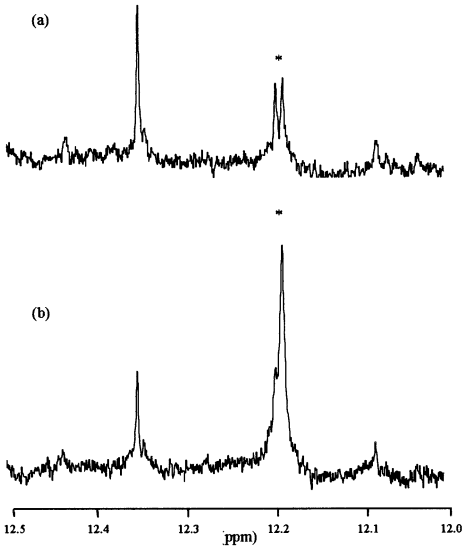


Figure 4. Selected region of the ^{1}H NMR spectrum of the acetone extract of *S. fruticosa* (a) at 300 K and (b) after the addition of 0.4 mmol of quercetin (spiking) at 300 K. The asterisk denotes the presence of the quercetin signal.

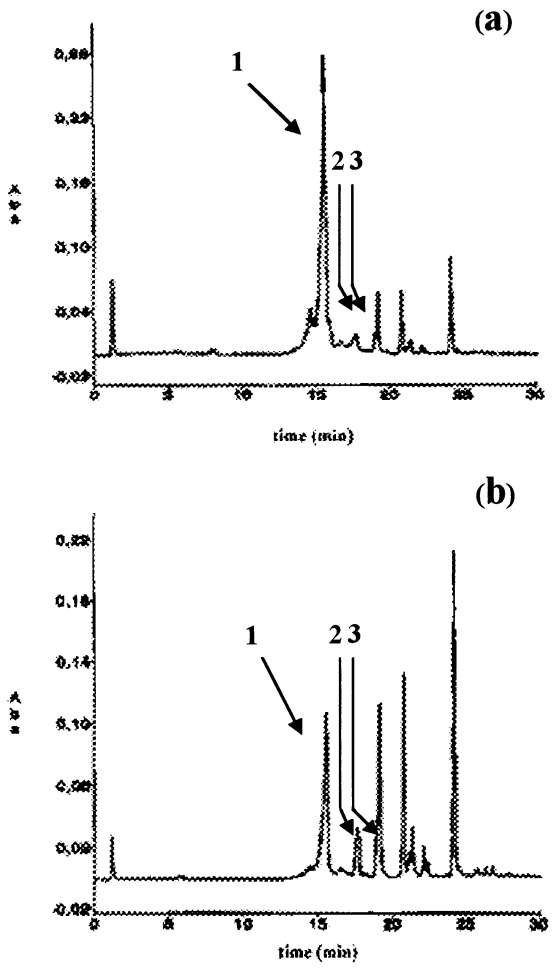


Figure 5. RP-HPLC profile of ethanol (a) and acetone (b) extracts of *S. fruticosa* at 334 nm. Peak assignment: rosmarinic acid, 1; quercetin or luteolin, 2; apigenin, 3.

intramolecular hydrogen bond. As a result it is possible to distinguish the flavones apigenin and luteolin from the flavonol quercetin.

It is important to define the temperature at which the ^{1}H NMR spectra of the extracts must be carried out in order to avoid undesirable broadening of the OH peaks. Therefore, detailed variable-temperature ^{1}H NMR spectra of the extracts in CD_3COCD_3 and CD_3OH solutions were obtained. With the use of the proper temperature and by spiking with standards, the two flavones (luteolin and apigenin) and quercetin were detected in ethanol and acetone extracts of Greek sage (**Figure 4**). In the ethanol extract of Greek oregano and summer savory none of the three flavonoids was detected by NMR, possibly due to their low concentration that was below the detection limit. Luteolin and apigenin were found in their acetone extracts. To the best of our knowledge this is the first application of the −OH spectral region for the analysis of real samples.

It can be concluded from the above that the flavonoids investigated have a rather small contribution to the total antioxidant activity of the extracts. The quantities of the flavonoids estimated by HPLC were insignificant, below the quantitative detection limits for apigenin, quercetin, or luteolin (0.1 μg/25 μL). In the case of Greek sage, less polar phenolic compounds that have been identified in other sage species (*33*) may be responsible for the high capacity of the acetone extracts (**Figure 5**; **Table 2**). For the rest of the plants the main active ingredient is rosmarinic acid, an important phytochemical, which has been found to be a potent active substance against human immunodeficiency virus type 1 (HIV-1) (*34*).

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