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Natural Antioxidant Constituents from Selected Aromatic Plants and Their Antimicrobial Activity Against Selected Pathogenic Microorganisms

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Summary

Aromatic plants contain natural antioxidant constituents such as phenolic compounds, which have attracted a great deal of public and scientific interest because of their health-promoting effects as antioxidants. Five plants, *Filipendula ulmaria* (meadow sweet), *Crataegus monogyna* (hawthorn), *Polygonum aviculare* (polygonum), *Potentilla anserina* (silverweed), and *Pelargonium purpureum* (little robin), have been examined in order to determine their phenolic composition. Reversed-phase high performance liquid chromatography (RP-HPLC) was employed for the identification and quantification of phenolic compounds of the aforementioned plants. Gas chromatography–mass spectrometry method (GC-MS) was also used for identification of phenolic compounds after silylation. Analysis of the non-volatile and thermolabile phenolic compounds by GC-MS presupposes their conversion into volatile and thermotolerant derivatives. The derivatization process was optimized against reagents, temperature and reaction time. The antioxidant capacity was determined in dried plants and in their methanol extracts with the Rancimat test using sunflower oil as substrate. Both pulverized plants and extracts showed antioxidant capacity. Total phenolic content in the extracts was determined spectrometrically applying the Folin-Ciocalteu assay and it ranged from 7.2 to 28.2 gallic acid equivalents (GAE)/(mg/mL). Antimicrobial activity of the extracts against selected microorganisms was performed using the disk diffusion method. Gram-(+) bacteria were more sensitive to the plant extracts than Gram-(–) bacteria.

Key words: plant antioxidants, RP-HPLC, GC-MS, trimethylsilyl derivatives, antimicrobial activity

Introduction

There is an intense interest in plant polyphenols as witnessed by the numerous papers devoted to various aspects of these compounds (*1*). The use of plants or herbs as antioxidants in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants (*2*). They tend to be water soluble, because they frequently occur combined as glycosides and they are usually located in the cell vacuole (*3*). Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (*4*). They also have metal chelation properties (*5*). Their significance in the human diet and antimicrobial activity has recently

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been established (*6*). The antioxidant properties of these compounds are often claimed for the protective effects of plant-based beverages against cardiovascular disease, certain forms of cancer and photosensitivity reactions (*7*). Recently, many separation techniques such as gas--liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have been proposed to separate and identify phenolic compounds (*8–10*). In the present study, the method of reversed-phase HPLC coupled with an ultraviolet--visible (UV/VIS) multiwavelength detector was used. This method enables the collection of on-line spectra and simultaneous quantification at several wavelengths. GC-MS was also employed for the analysis of major phenolic substances present in some aromatic plants in Greece because of the combination of the separation capabilities of GC and the power of MS as an identification and confirmation method. HPLC-MS is also a useful method for structural identification. The combination of the chromatographic method and mass spectrometry is very effective and elucidates better the structure of a particular compound. The object of this research is: (*i*) to determine the phenolic content of the examined plant extracts, (*ii*) to measure the antioxidant capacity of these extracts to inhibit lipid oxidation under accelerated conditions (Rancimat test), and (*iii*) to screen the antimicrobial activity of these plant extracts against some pathogenic microorganisms. There is very little research indicating the concentration of phenolic compounds in the examined aromatic plants, as well as their antioxidant capacity and antimicrobial activity.

Materials and Methods

Standards

Gallic acid, gentisic acid, *p*-coumaric acid, vanillic acid, ferulic acid, syringic acid, (+)-catechin, quercetin, apigenin, naringenin and myricetin were purchased from Sigma-Aldrich (Steinheim, Germany), and luteolin was from Roth (Karlsruhe, Germany). Caffeic acid was from Merck (Darmstadt, Germany), (–)-epicatechin was from Fluka AG (Buchs, Switzerland), and rutin was from Alexis Biochemicals (Lausen, Switzerland). Hydroxytyrosol, *p*-hydroxybenzoic acid and butylated hydroxytoluene (BHT) were a kind donation from the National Agricultural Research Foundation (N.AG.RE.F., Greece). Quantification was done *via* a calibration with standards (external standard method). All standards were prepared as stock solutions in methanol. Working standards were made by diluting stock solutions in 62.5 % aqueous methanol containing BHT 1 g/L, and 6 M HCL to yield concentrations ranging between 0.5–25 mg/mL. Working solutions of the standards were stored in darkness at –18 °C.

Solvents and reagents

All solvents and reagents from various suppliers were of the highest purity needed for each application. The Folin-Ciocalteu reagent and silylation reagents, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), were purchased from Merck (Darmstadt, Germany). For deactivating glassware surfaces, 5 % dimethyldichlorosilane (DMDCS) in toluene was obtained from Sigma-Aldrich (Steinheim, Germany).

Plant material

Samples (*Filipendula ulmaria*, *Crataegus monogyna*, *Polygonum aviculare*, *Potentilla anserina* and *Pelargonium purpureum*) were collected from Mount Ymittos, in continental Greece, at 350 m altitude in May 2005. Some samples were air-dried (at 25 °C in the dark) and some were lyophilized. All samples were analyzed within 3 months of collection.

Sample preparation and derivatization

The extraction method used for dried samples was as follows: 40 mL of 62.5 % aqueous methanol containing BHT (1 g/L) were added to 0.5 g of dried sample, then 10 mL of 6 M HCL were added and the mixture was stirred carefully. In each sample nitrogen was bubbled for approx. 40–60 s. The extraction mixture was then sonicated for 15 min and refluxed in a water bath at 90 °C for 2 h. The mixture was then either filtered and made up to 100 mL with methanol (*11*), then filtered quickly through a 0.45-μm membrane filter (Millex-HV) and injected into HPLC (Jasco, Japan), or extracted with 30 mL (3×10 mL) of ethyl acetate. The organic layer was collected, reduced to 10 mL by rotary evaporation (37 °C) and centrifuged for 10 min. Anhydrous Na_2SO_4 was then added to remove residual moisture. Then, 100 μL of the organic layer were derivatized after evaporation of the solvent under nitrogen stream. For the silylation procedure, a mixture of TMCS (100 μL) and BSTFA (200 μL) was added and vortexed in screw capped glass tubes (previously deactivated with 5 % DMDCS in toluene, rinsed twice with toluene and three times with methanol), and consecutively placed in a water bath at 80 °C for 45 min. From the silylated mixture, 1 μL was directly analyzed by GC-MS (Fisons, Italy).

HPLC analysis

The analytical HPLC system employed consisted of a JASCO high-performance liquid chromatograph coupled with a UV/VIS multiwavelength detector (MD-910 JASCO). The analytical data were evaluated using a JASCO data processing system (DP-L910/V). The separation was achieved on a Waters Spherisorb® 5 μm ODS2 4.6×250 mm column at ambient temperature. The mobile phase consisted of water with 1 % glacial acetic acid (solvent A), water with 6 % glacial acetic acid (solvent B), and water-acetonitrile (volume ratio=65:30) with 5 % glacial acetic acid (solvent C). The gradient used was: 100 % A 0–10 min, 100 % B 10–30 min, 90 % B/10 % C 30–50 min, 80 % B/20 % C 50–60 min, 70 % B/30 % C 60–70 min, 100 % C 70–105 min, 100 % A 105–110 min; post time 10 min before next injection. The flow rate was 0.5 mL/min and the injection volume was 20 μL. The monitoring wavelength was 280 nm for the phenolic acids and 320 and 370 nm for flavones and flavonoles, respectively. The identification of each compound was based on a combination of retention time and spectral matching.

GC-MS analysis

The silylated samples were injected into a GC-MS system consisting of a Fisons GC 8000 Series, model 8060 gas chromatograph coupled with a Fisons MD 800 mass spectrometer in the EI (electron impact) mode with the electron energy set at 70 eV and the mass range of m/z=25–700. A capillary column low-bleed CP-Sil™ 8 CB-MS (30 m×0.32 mm, i.d.), with 0.25 μm film thickness of coated material was used. The injector was set at 280 °C and the detector at 290 °C. GC was performed in the splitless mode with 1 min splitless time. The stepped temperature program was as follows: from 70 to 135 °C with 2 °C/min, held for 10 min, from 135 to 220 °C with 4 °C/min, held for 10 min, from 220 to 270 °C with 3.5 °C/min and then held for 20 min. A post run of 10 min at 70 °C was sufficient for the next injection. The flow rate of the carrier gas (helium) was maintained at 1.9 mL/min. Identification of compounds was obtained by comparing the retention times with those of authentic compounds and the spectral data obtained from the Wiley and NIST libraries. Each determination was carried out in duplicate.

Antioxidant capacity (Rancimat test)

Samples of sunflower oil (3.5 g) containing 0.02 % of extract or 2 % of ground material were subjected to oxidation at 110 °C (air flow 20 L/h). Induction periods (IP/h) were recorded automatically. The protection factors (PF) were calculated according to the following equation:

$$PF=IP_{extract}/IP_{control} \qquad /1/$$

according to Exarchou *et al.* (*12*).

Determination of total phenolic content of plant extracts

Total phenolic content was measured by the Folin-Ciocalteu assay (*13*). Quantification was performed with the hydrolysed samples. Results were expressed as gallic acid equivalents (GAE)/(mg/mL). A calibration curve of absorbance *vs.* concentration of gallic acid was used to derive the GAE concentrations for plant extracts.

Antimicrobial assay

The methanol extracts were tested against a panel of pathogenic microorganisms, including *Escherichia coli* O157:H7 NCTC 12900, *Salmonella enteridis* PT 4, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* Scott A, *Pseudomonas putida* AMF 178 and *Bacillus cereus* FSS 134. Microorganisms were stored frozen in bead vials (Protect, Technical Service Consultants Ltd., Heywood, Lancashire, UK). Resuscitation of bacterial strains was carried out in 10 mL of brain-heart (BH) broth (Merck cat. no. 1.10493) incubated overnight at 37 °C for *E. coli* and *S. enteridis*, at 35 °C for *B. cereus*, at 30 °C for *L. monocytogenes* and *S. aureus* and at 25 °C for *P. putida*. Resuscitated cultures were diluted tenfold in Ringer's solution (Lab M, UK) for the inoculation of 10 mL of BH broth (Merck cat. no. 1.10493) to yield an initial suspension of approx. 10 to 100 CFU/mL. All broths were then incubated statically at the aforementioned temperatures for each microorganism for 18–24 h to guarantee that all cells were in the stationary phase. Susceptibility of the tested organism to the extract was determined by employing the standard disk diffusion technique. The bacterial suspensions were diluted tenfold in Ringer's solution (Lab M, UK), and 0.1 mL of the appropriate dilution was spread on BH agar plates (Merck cat. no. 1.13825) in order to give a population of approx. 10^6 CFU/plate. Sterile paper discs with a diameter of 6.48 mm (Whatman no. 2) were placed onto the inoculated agar surface. A volume of 5 μL of each plant extract was added to the paper discs. Each experiment was carried out in triplicate. Petri dishes were incubated for 48 h at 37 °C for *E. coli* and *S. enteridis*, at 35 °C for *B. cereus* and at 30 °C for *L. monocytogenes* and *S. aureus* and at 25 °C for *P. putida*. After incubation, the inhibition zones were estimated by taking photographs of the Petri dishes with a Sony camera (Xwave HAD SSC-DC50AP) and processed using the Impuls Vision XL 2.5 software. Each inhibition zone diameter was measured three times (three different plates) and the average value was calculated.

Results and Discussion

Antioxidant capacity

The antioxidant capacity (expressed as PF values) and the total phenolic content of all extracts are shown in Table 1. The concentration of total phenolics varied slightly in the plant material and ranged from 7.2 to 28.2 GAE/(mg/mL). The highest concentration was found in *Pelargonium purpureum*, and the lowest in *Filipendula ulmaria*. Similar concentration of plant phenolics from herbs and medicinal plants collected in Finland has been reported recently (*13*).

Table 1. Total phenolics in plant extracts and their antioxidant capacity (expressed as PF values)

Plant	Part examined	Drying method[a]	γ(GAE*)[b]/(mg/mL)	PF (ground material)	PF (methanol extracts)
Filipendula ulmaria	Leaves	Air	7.2±0.3	1.3	1.2
Crataegus monogyna	Herb	Air	10.5±0.3	1.5	1.4
Polygonum aviculare	Flower	F/v	8.6±0.2	1.0	0.8
Potentilla anserina	Leaves	F/v	18.4±0.3	1.8	1.7
Pelargonium purpureum	Leaves	F/v	28.2±0.1	3.1	2.9

[a]Air=air drying; F/v=freeze-vacuum drying, *i.e.* lyophilization; [b]Mean of duplicate assays; PF=protection factor

*GAE=total phenolics in gallic acid equivalents

The outcome of the Rancimat test supports the hypothesis that aromatic plants are good sources of natural antioxidants such as phenolic compounds and when it is done accurately, it offers an efficient, simple and automated assay. When ground material was added to sunflower oil, protection factors were slightly higher compared to the addition of methanol extracts. Similar PF values for ethanol and acetone extracts from plants of Greek origin have been reported (*12*).

HPLC analysis

HPLC with UV/VIS multiwavelength detector was used since all phenolic compounds show intense absorption in the UV region of the spectrum. The present method is simple, easy to use, and effective enough for identification and quantification of major phenolic compounds in aromatic plants. A similar technique has been reported by other authors for the analysis of major flavonoid aglycones (*14*). Spherisorb® ODS2 stationary phase, which was used in this study to separate phenolic acids and flavonoids in the aforementioned wavelengths of aromatic plants, produced satisfactory results. After extraction and acid hydrolysis, the content of phenolic substances was determined. Quantification was done *via* a calibration with standards (external standard method). The content of phenolic acids detected in the analysed samples is shown in Table 2. Additionally, the content of flavonoids identified in the same plant extracts is shown in Table 3. Results are expressed as mg/L of plant extract. Another phenolic compound which was detected in some samples was hydroxytyrosol. Hydroxytyrosol (also known as dihydroxyphenylethanol) is a main bioactive constituent of olives (*15*). Only *Filipendula ulmaria* contained this compound (78 mg/mL of plant extract). In all the other plant extracts hydroxytyrosol was detected in traces. The most abundant phenolic acids were ferulic (104–169 mg/mL of plant extract) and caffeic acid (64–200 mg/mL of plant extract). (+)-Catechin, rutin (quercetin 3-*O*-rhamnose glycoside) and quercetin were the most abundant flavonoids. Myricetin was detected only in *Filipendula ulmaria*. The levels of phenolic compounds were as expected according to previous investigations of these compounds in similar herbs or plants (*16*). The data presented in Tables 1–3 are considered as indicative of phenolic content of these aromatic plants. Data about most of the examined plant extracts are very scarce in the literature. Among others, time of harvest and storing conditions are considered responsible for the observed variations in the phenolic contents.

GC-MS analysis

The isolation and quantification of phenolic compounds from plant material is difficult because of their chemical complexity. The sensitivity and resolving power of capillary GC-MS make this technique particularly suitable for unambiguous detection of phenolic compounds in the hydrolysed samples. Silylation is the most widely used derivatization procedure for sample analysis by GC. The derivatives are generally less polar, more volatile and more thermally stable. Trimethylchlorosilane (TMCS) is a silylation catalyst, rarely used alone but typically mixed with other silylation reagents to increase their reactivity in derivatization. Several variables were examined to determine their roles in the derivatization procedure: (*i*) temperature, (*ii*) reaction time, and (*iii*) the concentration of silylating reagent required to complete the derivatization. In our study the temperature and re-

Table 2. Content of phenolic acids in the examined plant extracts

Plant	γ(plant extract)*/(mg/L)							
	gallic acid	gentisic acid	caffeic acid	*p*-coumaric acid	vanillic acid	syringic acid	ferulic acid	*p*-hydroxy-benzoic acid
Filipendula ulmaria	49±0.3	ND	135±0.2	139±0.4	185±0.2	ND	169±0.4	ND
Crataegus monogyna	ND	ND	81±0.1	66±0.2	ND	ND	123±0.3	ND
Polygonum aviculare	ND	ND	ND	ND	ND	ND	ND	ND
Potentilla anserina	ND	ND	64±0.2	ND	ND	ND	104±0.3	ND
Pelargonium purpureum	140±0.2	32±0.3	200±0.3	41±0.2	20±0.2	11±0.2	ND	54±0.1

*Each value is the mean (mg/L plant extract) of two replications±standard deviation; ND=not detected

Table 3. Flavonoid content in the examined plant extracts

Plant	γ(plant extract)*/(mg/L)							
	quercetin	apigenin	luteolin	naringenin	myricetin	rutin	(+)-catechin (hydrated)	(–)-epicatechin
Filipendula ulmaria	ND	ND	ND	ND	520±0.9	ND	19±0.1	ND
Crataegus monogyna	ND	ND	ND	ND	ND	100±0.3	ND	ND
Polygonum aviculare	ND	ND	91±0.2	ND	ND	71±0.2	19±0.1	28±0.1
Potentilla anserina	73±0.2	ND	ND	ND	ND	23±0.1	ND	ND
Pelargonium purpureum	112±0.2	ND	ND	ND	ND	45±0.1	15±0.1	56±0.2

*Each value is the mean (mg/L plant extract) of two replications±standard deviation; ND=not detected

action time used were sufficient for the silylation of phenolic compounds. Apigenin, luteolin, naringenin and myricetin displayed very poor sensitivity to the derivatization process even at concentrations as high as 30 mg/mL. In most cases, 1 % TMCS in BSTFA is sufficient to achieve the desired derivatization. In a previous report, a large excess of the derivatization reagents BSTFA and TMCS was used for the determination of phenolic antioxidants in green tea (*8*). In the present study, BSTFA and TMCS were used in excess to ensure that the silylation was complete. Care was taken to ensure anhydrous conditions during the derivatization process because of the high sensitivity of trimethylsilyl (TMS) derivatives to moisture. Therefore, anhydrous Na_2SO_4 was added. The GC oven temperature program as well as the injector and detector temperatures were based on previous experience with the analysis of marker compounds in *Ginkgo biloba* L. extract (*10*). After derivatization, and prior to employing GC-MS for the determination of phenolic compounds in plant extracts, a standard mixture of all substances was tested. Data obtained showed excellent resolution between all compounds of interest. Molecular mass (MM) and important ions present in the mass spectra of silylated phenolic compounds in the examined plant extracts are presented in Table 4.

Antimicrobial activity

Antimicrobial assays described in the literature include measurements of: (*i*) the radius or diameter of the zone of inhibition of bacterial growth around paper disks impregnated with (or wells containing) an antimicrobial compound on agar media, (*ii*) the inhibition of bacterial growth on an agar medium with the antimicrobial compound diffused in the agar, (*iii*) the minimum inhibitory concentration (MIC) of the antimicrobial compound in liquid media, and (*iv*) the changes in absorbance or impedance in a liquid growth medium containing the antimicrobial compound. To screen the antimicrobial activity of 'unknown' compounds, the second methodology mentioned above is considered to be the simplest because the results are obtained rapidly. Vardar-Ünlü *et al.* (*17*) used the disk diffusion method to determine the antimicrobial activity of the essential oil and methanol extracts using several microbial strains. The antimicrobial activity of plant extracts is shown in Table 5. The

Table 4. Molecular mass (MM) and important ions present in the mass spectra of silylated phenolic compounds* in the examined plant extracts by GC-MS

Compound	MM (Silylated compounds)	Identified ions (*m/z*)
p-Hydroxybenzoic acid	282	267 (100 %), 193, 223, 282
Vanillic acid	312	149 (100 %), 312, 223, 165
Gentisic acid	370	355 (100 %), 281, 147, 223, 267, 370
Gallic acid	458	281 (100 %), 458, 179, 147
p-Coumaric acid	308	219 (100 %), 293, 308, 249
Ferulic acid	338	338 (100 %), 308, 323, 249, 293, 219, 279
Caffeic acid	396	219 (100 %), 396, 381, 191
Quercetin	647	575 (100 %), 647, 487
(+)-Catechin	650	368 (100 %), 355, 650, 267, 383, 179, 297
Hydroxytyrosol	370	267 (100 %), 193, 179, 370
Syringic acid	342	327 (100 %), 312, 297, 342
(–)-Epicatechin	650	368 (100 %), 355, 267, 147, 650
p-Hydroxyphenylacetic acid	296	179 (100 %), 164, 149, 296
Protocatechuic acid	370	193 (100 %), 223, 370, 267
Cinnamic acid	220	131 (100 %), 205, 103, 161, 220

*Identified as trimethylsilyl (TMS) derivatives

Table 5. Antimicrobial activity of plant extracts (V(sample)=5 μL; N=3)

Plant extracts	*Escherichia coli* O157:H7 NCTC 12900	*Salmonella enteridis* PT 4	*Staphylococcus aureus* ATCC 6538	*Listeria monocytogenes* Scott A	*Bacillus cereus* FSS 134	*Pseudomonas putida* AMF 178
Filipendula ulmaria	–	–	~	++	~	+
Crataegus monogyna	~	–	–	~	~	~
Polygonum aviculare	–	–	–	~	–	–
Potentilla anserina	–	–	–	+	–	+
Pelargonium purpureum	++	–	+	++	+	++

– No antimicrobial capacity, internal zone (i.z.) of sample<i.z. of solvent (62.5 % aqueous methanol); ~ slight antimicrobial capacity, i.z. of sample 1–3 mm>i.z. of solvent; + moderate antimicrobial capacity, i.z. of sample 3–4 mm>i.z. of solvent; ++ clear antimicrobial capacity, i.z. of sample 4–10 mm>i.z. of solvent

plant extracts found to be more effective in inhibiting the microorganisms tested were *Pelargonium purpureum* and *Filipendula ulmaria. Listeria monocytogenes* Scott A was the most sensitive microorganism to the plant extracts examined in this study. On the contrary, all the extracts failed to inhibit *S. enteridis* PT 4. The results in Table 5 revealed that Gram-(+) bacteria are more sensitive to the plant extracts than Gram-(–) bacteria, especially the Enterobacteriaceae (*E. coli* O157:H7 NCTC 12900 and *S. enteridis* PT 4). However, similar inhibition of *L. monocytogenes* has also been observed for *P. putida* AMF 178. More literature data about the antioxidant activity of herbs against microorganisms is available (*18–20*).

Conclusions

The outcome of this study shows that plant extracts contain polyphenols (natural antioxidants), which are believed to be effective nutrients in the prevention of oxidative stress-related diseases such as cancer and heart diseases. These extracts, possibly mainly due to their phenolic content, retard oxidative degradation of lipids, as it is shown by the Rancimat test. The Folin-Ciocalteu assay, which was used to determine the total phenolic compounds, has for many years been used as a measure of total phenolics in natural products, but the basic mechanism is an oxidation/reduction reaction and, as such, can be considered as another method to determine antioxidant capacity. The influence of seasonal harvest of plants, geographical location and altitude, storage and extraction procedures still has to be investigated in detail in order to be able to draw the utmost benefit for nutritional and even more for industrial use. This experiment also proved that silyl derivatization offers a very good alternative for the identification of phenolic compounds instead of HPLC, which is usually the most preferred method for the analysis of polyphenols. However, it should be stressed that more research is needed towards the identification of silyl derivatives.

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References

1. D. Tura, K. Robards, Sample handling strategies for the determination of biophenols in food and plants, *J. Chromatogr. A, 975* (2002) 71–93.
2. H.L. Madsen, G. Bertelsen, Spices as antioxidants, *Trends Food Sci. Technol. 6* (1995) 271–277.
3. J.B. Harborne: Phenolic Compounds. In: *Phytochemical Methods, A Guide to Modern Techniques of Plant Analysis*, Chapman and Hall, London, UK (1998) pp. 40–106.
4. P.G. Pietta, Flavonoids as antioxidants, *J. Nat. Prod. 63* (2000) 1035–1042.
5. C.A. Rice-Evans, N.J. Miller, G. Paganga, Antioxidant properties of phenolic compounds, *Trends Plant Sci. 2* (1997) 152–159.
6. J.P. Rauha, S. Remes, M. Heinonen, A. Hopia, M. Kähkönen, T. Kujala, K. Pihlaja, H. Vuorela, P. Vuorela, Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds, *Int. J. Food Microbiol. 56* (2000) 3–12.
7. L. Bravo, Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance, *Nutr. Rev. 56* (1998) 317–333.
8. Y. Zuo, H. Chen, Y. Deng, Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector, *Talanta, 57* (2002) 307–316.
9. T. Chu, C. Chang, Y. Liao, Y. Chen, Microwave-accelerated derivatization process for the determination of phenolic acids by gas chromatography–mass spectrometry, *Talanta, 54* (2001) 1163–1171.
10. F. Deng, S.W. Zito, Development and validation of a gas chromatographic-mass spectrometric method for simultaneous identification and quantification of marker compounds including bilobalide, ginkgolides and flavonoids in *Ginkgo biloba* L. extract and pharmaceutical preparations, *J. Chromatogr. A, 986* (2003) 121–127.
11. U. Justesen, P. Knuthsen, T. Leth, Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection, *J. Chromatogr. A, 913* (1998) 101–110.
12. V. Exarchou, N. Nenadis, M. Tsimidou, I.P. Gerothanasis, A. Troganis, D. Boskou, Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory, *J. Agric. Food Chem. 50* (2002) 5294–5299.
13. M.P. Kähkönen, A.I. Hopia, J.V. Heikki, J.P. Rauha, K. Pihlaja, T.S. Kujala, M. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, *J. Agric. Food Chem. 47* (1999) 3954–3962.
14. P. Mattila, J. Astola, J. Kumpulainen, Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections, *J. Agric. Food Chem. 48* (2000) 5834–5841.
15. Y. O'Dowd, F. Driss, P. My-Chan Dang, C. Elbim, M.A. Gougerot-Pocidalo, C. Pasquier, J. El-Benna, Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: Scavenging of hydrogen peroxide but not superoxide anion produced by human neutrophils, *Biochem. Pharmacol. 68* (2004) 2003–2008.
16. W. Zheng, S.Y. Wang, Antioxidant activity and phenolic compounds in selected herbs, *J. Agric. Food Chem. 49* (2001) 5165–5170.
17. G. Vardar-Ünlü, F. Candan, A. Sökmen, D. Daferera, M. Polissiou, M. Sökmen, E. Dömnez, B. Tepe, Antimicrobial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey. var. *pectinatus* (Lamiaceae), *J. Agric. Food Chem. 50* (2003) 63–67.
18. C.C. Tassou, G.J.E. Nychas, P.N. Skandamis: Herbs and Spices and Antimicrobials. In: *Handbook of Herbs and Spices*, K.V. Peter (Ed.), Woodhead Publishing Ltd., Cambridge, UK (2004) pp. 22–40.
19. C.C. Tassou, K. Koutsoumanis, G.J.E. Nychas, Inhibition of *Salmonella enteridis* and *Staphylococcus aureus* in nutrient broth by mint essential oil, *Food Res. Int*. *33* (2000) 273–280.
20. C.C. Tassou, G.J.E. Nychas, Inhibition of *Staphylococcus aureus* by olive phenol in broth and in model food system, *J. Food Prot*. *57* (1994) 120–124.