

Antimicrobial and Free Radical Scavenging Activities of Basil (*Ocimum basilicum*) Essential Oil Isolated from Five Plant Varieties Growing in Greece

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Abstract

The antimicrobial activity and DPPH free radical scavenging capacity of six essential oils isolated from the leaves (in all cases) and flowers (in one case) of five basil varieties (*Ocimum basilicum*) growing in Greece were examined. The antimicrobial activity was probed via disc diffusion and minimum inhibitory concentration and tested against 16 Gram-positive and 17 Gram-negative ATCC bacterial strains. The essential oils from three basil varieties exhibited statistically significant increased antimicrobial activity towards Gram-positive relative to Gram-negative bacteria. Both antimicrobial and free radical scavenging activities showed significant dependence on basil variety as well as on plant part.

Keywords: Basil; *Ocimum basilicum*; Essential oil; Antimicrobial activity; Antioxidant activity

Abbreviations: DPPH: 2,2-diphenyl-1-picrylhydrazyl; EO: Essential Oil; Trolox: 6-hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid; S: Small-leaved basil; L: Large-leaved basil; Le: Lettuce-leaved basil; C: Cinnamon basil; R: Red basil; F: Flowers of lettuce-leaved basil

Introduction

Although new technologies (such as genetic engineering, irradiation of food and modified-atmosphere packaging) can improve food safety, microbiological hazards and the foodborne diseases they cause may lead to increasingly important public health problems. Over the past decades, a significant increase in the incidence of diseases caused by microorganisms transmitted mainly by food (such as *Salmonella* spp. and *Campylobacter* spp.) has been reported [1]. The emergence of increased antibiotic resistance in bacteria causing foodborne disease is an additional complicating factor. In fact, there is shortage of new antibiotics able to act against multidrug-resistant bacteria [2] and thus innovative approaches are needed for the development of new antimicrobial agents. Some areas currently being investigated include natural products screening, exploring novel chemical species and development of antibiotic potentiators (e.g. efflux-pump inhibitors).

In recent years, essential oils and herbal extracts have attracted scientific interest due to their potential use as antimicrobial and/or antioxidant compounds in food [3-5]. Essential oils (EOs) are the volatile oily liquid products of the secondary metabolism of aromatic plants, obtained from different plant parts. Several EOs have been studied for several types of biological effects among which antimicrobial, anti-inflammatory and antioxidant activities [6-8]. In fact, there is a renewed interest for the possible commercial applications of EOs in sectors like the food and pharmaceutical industries. Concentrating in the food industry, there is a growing consumers' demand for the development of natural food additives, which will supply protection against spoilage, food-borne pathogens and oxidation processes thus reducing the use of synthetic food additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). There is experimental evidence,

however not definitive [9], which associates the use of such additives with toxic effects in biological systems [10].

Basil (*Ocimum basilicum*) is an aromatic plant used extensively to add aroma and flavor in food. Traditionally, basil has been used as a medicinal plant. The essential oil of the species contains a wide and varying array of chemical compounds, depending on variations in genotype, climatic conditions, geographical location, seasonal variation, agronomic treatment and stage of development [11-16]. Similar variation in chemical composition has been documented in the phenolic acid constituents present in extracts from *O. basilicum* accessions [17-19]. An often used classification of basil based on its EO composition and according to geographical origin, involves the following four major chemotypes [11], even though several chemotype variations have also been reported [14,16]: (i) European chemotype with EO having linalool and estragole as major components, (ii) Reunion chemotype with EO containing estragole as main component, (iii) Tropical chemotype with methylcinnamate as main component and (iv) Eugenol chemotype containing eugenol as main component.

The antimicrobial activity of basil EOs and herb extracts isolated from different *Ocimum* species has been studied by several groups. In some cases, the reaction of basil EOs with both Gram-positive and Gram-negative bacteria has been probed and effort has been made to correlate the expressed antimicrobial activity with the EO chemical composition [4,15,20-24].

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Similarly, there have been several studies aiming at probing the antioxidant activity of basil EOs and extracts derived from different *Ocimum* species with different methods [3,15,19,24-30].

In the current study, the determination of *in vitro* antimicrobial activity and of the free-radical scavenging activity of the EOs of five basil varieties growing in Greece is undertaken. More specifically, research was carried out on the EO obtained from the leaves of small-leaved (S), large-leaved (L), lettuce-leaved (Le), cinnamon (C) and red (R) basil, as well as on the EO obtained from the flowers of lettuce-leaved basil (F). So far, there have been only few studies related with basil EOs or solvent extracts growing in Greece. The most relevant studies include the following: a report on the antioxidant properties of acetic and methanolic extracts of basil grown on the island of Crete [31], a report on the antimicrobial properties of basil essential oil against 24 clinical isolates of *Staphylococcus aureus* [32], and a study on the effects of individual selection on "Greek basil" EO composition [33]. Besides the geographical interest, the current study examines the *in vitro* antimicrobial activity of basil EO in a large number of bacterial strains (33 in total) which are almost equally distributed between Gram-positive and Gram-negative ones (16 and 17 strains respectively) for the first time. This allows for more accurate comparisons of the expressed antimicrobial activities between the two groups of bacteria as well as between the different basil varieties with statistical methods. In addition, in the current study an effort is made to gain insight on the dependence of the properties of basil EO on the part of the plant used for its isolation (leaves vs. flowers) for one specific plant variety (lettuce-leaved basil). Finally, effort is made to probe for possible correlation between the antimicrobial and antioxidant activities.

Materials and Methods

Plant material

Essential oil (EO) was obtained from the leaves of five basil varieties (*Ocimum basilicum*) namely small-leaved (S), large-leaved (L), lettuce-leaved (Le), cinnamon (C) and red (R) basil, as well as on the EO obtained from the flowers of lettuce-leaved basil (F). The seeds of the above mentioned five varieties of *Ocimum basilicum* were obtained from the Seed Bank of the Technological Educational Institute (TEI) of Ionian Islands (Department of Food Technology, Argostoli, Kefalonia, Greece). All basil plants were grown at the premises of TEI of Ionian Islands at Kefalonia island, under the same conditions (climate, soil, agronomic treatment). All plants were planted and collected at same time of the year (early spring and mid-summer respectively). The lettuce-leaved basil variety was the only one that during the time of collection had large amounts of flowers and for this reason EO was isolated separately from leaves and flowers.

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid (Trolox) were obtained from Sigma. Ethanol absolute was obtained from MERCK.

Essential oil extraction

One Kg of plant material (leaves or flowers) and 500 ml water were placed in a hydro-distillation apparatus (Albrigi Luigi S.R.L.) and the EO was isolated for 3 hours, following the manufacturer's instructions. The obtained EO was separated, dried over anhydrous sodium sulfate, and stored under nitrogen in a sealed vial at -20°C before usage. The voucher specimen of the EO was deposited in the Institutional Bank of EOs, TEI of Ionian Islands, Kefalonia, Greece.

Microbial strains

All strains of bacteria were obtained from the American Type Culture Collection (ATCC). Bacterial cultures were maintained in Trypticase Soy broth/agar (Difco-BD). The following strains were used: *Salmonella arizonae* ATCC 13314, *S. typhimurium* ATCC 14028, *S. schottmuelleri* ATCC 8759, *S. tallahassee* ATCC 12002, *S. enteritidis* ATCC 13076, *S. typhimurium* ATCC 13311, *S. pullorum* ATCC 19945, *Klebsiella pneumoniae* ATCC 13883, *K. pneumoniae* ATCC 10031, *Escherichia coli* ATCC 11303, *Enterobacter aerogenes* ATCC 35028, *Proteus vulgaris* ATCC 6380, *P. vulgaris* ATCC 49132, *Shigella boydii* ATCC 9207, *S. sonnei* ATCC 9290, *S. flexneri* ATCC 12022, *Yersinia enterocolitica* ATCC 23715, *Staphylococcus aureus* ATCC 12598, *S. aureus* ATCC 25923, *S. aureus* ATCC 12600, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 14990, *S. lentus* ATCC 700403, *S. hominis* ATCC 27844, *S. saprophyticus* ATCC 35552, *Micrococcus luteus* ATCC 533, *M. luteus* 49732, *M. luteus* ATCC 4698, *Listeria monocytogenes* ATCC 15313, *L. monocytogenes* ATCC 7644, *L. monocytogenes* 19111, *Bacillus cereus* ATCC 14579, *B. cereus* ATCC 10876.

Determination of antimicrobial activity

The agar disc diffusion method (Disc assay, DA) was employed for screening the antimicrobial activity of basil EOs [34]. The test was performed in sterile Petri dishes (150 mm diameter) containing TSA medium (Difco-BD). Briefly, suspension in Trypticase Soy Broth (TSB) of the tested microorganism (2 ml of 10⁶ CFU/ml) was added to 18 ml soft agar (Noble Agar, Difco-BD) and placed onto TSA plates, for all bacterial strains. Sterile filter paper discs (4 mm diameter) were placed on the surface of the media previously inoculated and 5 µl of essential oil were pipetted onto the disc. One filter paper disc was placed per Petri dish to avoid possible cross-reactivity among the different oils. Every dish was sealed with parafilm to avoid evaporation and incubated at 37 °C for 24 hours. Negative controls were prepared using discs without any oil. The diameter of the inhibition zone (expressed in mm) was used as a measure of the antimicrobial activity of the tested EOs. All tests were performed in triplicate. The scale of measurements was the following: Diameter of inhibition zone (DIZ) >15 mm strongly inhibitory EO, DIZ between 10-15 mm moderately inhibitory EO and DIZ between 5-9 mm weakly inhibitory EO.

Minimum inhibitory concentration (MIC) was determined for all EOs using sterile 96-well microplates, based on a standard NCCLS method [35]. 190 µl of cell suspension (10⁵ CFU/ml) in TSB was placed in the first well to which 10 µl of EO were added resulting in a final concentration of the first well equal to 50 µl/ml. In the rest of the wells, 100 µl of the cell suspension was added and serial twofold dilutions were performed thus achieving a final concentration range from 0.20 to 50 µl/ml (which is divided in the following nine levels: 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 12.5, 25.0 and 50.0 µl/ml). The last well of each row was used as negative control containing only 100 µl of cell suspension. Erythromycin (5 µg/ml) was used as a positive reference standard. Microplates were incubated at 37 °C for 24 hours for all bacterial strains. All tests were performed in triplicate. MIC was determined as the lowest concentration of the test samples where no visible changes were detected in the broth medium. To verify results, the MIC oil concentration and the one prior to it were repeated in separate plates to ensure the lack of EO vapor influence on neighboring wells. In all tests, plates were sealed with parafilm to avoid evaporation.

Determination of free radical scavenging activity

The antioxidant activity of the EOs was probed via their ability

to interact with the free radical DPPH by reducing it to DPPH-H (hydrogen donating or radical scavenging ability) [36]. A control solution of 100 μM DPPH radical in ethanol was prepared and displayed an absorbance maximum at 517 nm. The EO under study was added to the freshly prepared control ethanolic solution of DPPH producing a solution of x% v/v in EO (x=0.1 either 0.005 or 0.001). Subsequently, the time course of the decay of the 517 nm absorption band of the DPPH radical was observed for a time period of 2 hours to allow for an almost complete reaction of the EO with the DPPH radical. It is noted that the need for using different EO dilution factors is due to the differential free radical scavenging activity expressed by the EOs. However, this fact is accounted for in the calculation of the total antioxidant activity of each EO (see below) which is expressed per ml of (non-diluted) essential oil and is not dependent on the EO dilution factor employed during the experimental procedure. All measurements were performed in triplicate, in a Shimadzu UV 2100 UV-VIS spectrophotometer at room temperature. All absorbance readings were made against a blank containing solely ethanol. The 100 μM DPPH ethanolic solution was always freshly prepared and its absorbance at 517 nm was measured both at the beginning and the end of each run, in order to ensure equivalent experimental conditions and the absence of any decomposition of the control solution. In all six EOs, a double exponential function (shown in the following equation 1) was needed in order to provide a satisfactory fit to the experimental data points (DPPH absorbance at 517 nm vs. time):

$$I(t) = I_f + A_1 \cdot \exp(-t/t_1) + A_2 \cdot \exp(-t/t_2) \quad (1)$$

where I_f , A_1 , t_1 , A_2 and t_2 were used as fitting parameters. The symbols in Equation 1 have the following meanings: $I(t)$ are the absorbance readings as a function of time, I_f is the final absorbance value reached after an “infinite” amount of time when the overall antioxidant activity of the EO has been expressed, t_1 and t_2 are the time constants of the two single exponentials and A_1 , A_2 are the amplitudes of each separate single exponential. It is noted that in all experiments a quantity of 3 μl of EO (either pure or pre-diluted by a factor 20 or 100) was added to 3 ml of the control 100 μM DPPH ethanolic solution thus producing an infinitesimal dilution of the latter (by a factor of 0.099%). For this reason, and by taking into account that the EO itself displays zero absorbance at 517 nm, the absorbance reading at $t=0$ is set equal to the one of the control 100 μM DPPH ethanolic solution. The magnitude of the parameter I_f is used subsequently in order to quantify the EO’s antioxidant activity in terms of Trolox equivalents (TEAC: Trolox equivalent antioxidant capacity). Trolox is a water-soluble analog of vitamin E and its antioxidant properties are well established. A Trolox calibration curve was produced by measuring the absorbance of a 100 μM DPPH control ethanolic solution at 517 nm after addition of Trolox at increasing concentrations (0-40 μM). The absorbance readings were obtained after the completion of the reaction of Trolox with the DPPH radical which is quite fast (much faster than the reaction of the EO) and occurred within 20 ± 5 min after Trolox addition. The use of Trolox calibration curve for deriving TEAC values has been employed by Tzika et al. [37] for probing the antioxidant properties of fruits and vegetable shots and juices via the reduction of the TEMPOL radical. The resulting Trolox calibration curve is given below and also shown in Figure 1:

$$y = -0.024 \cdot c + 1.36 \quad (2)$$

where c is the Trolox concentration (in μM) and y is the absorbance reading at 517 nm. As shown in Figure 1, there is an excellent linear fit ($R^2=0.992$) in the whole range of absorbance readings (1.36 – 0.37) and Trolox concentrations (0-40 μM) employed. By substituting the value

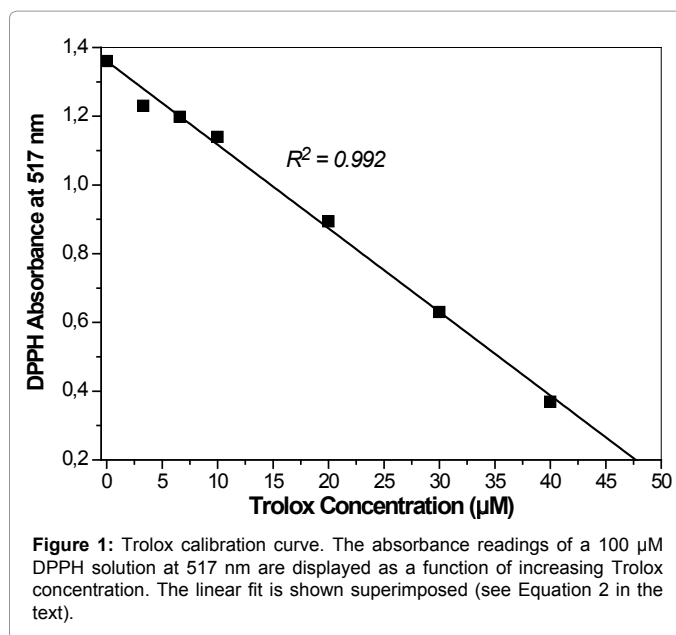


Figure 1: Trolox calibration curve. The absorbance readings of a 100 μM DPPH solution at 517 nm are displayed as a function of increasing Trolox concentration. The linear fit is shown superimposed (see Equation 2 in the text).

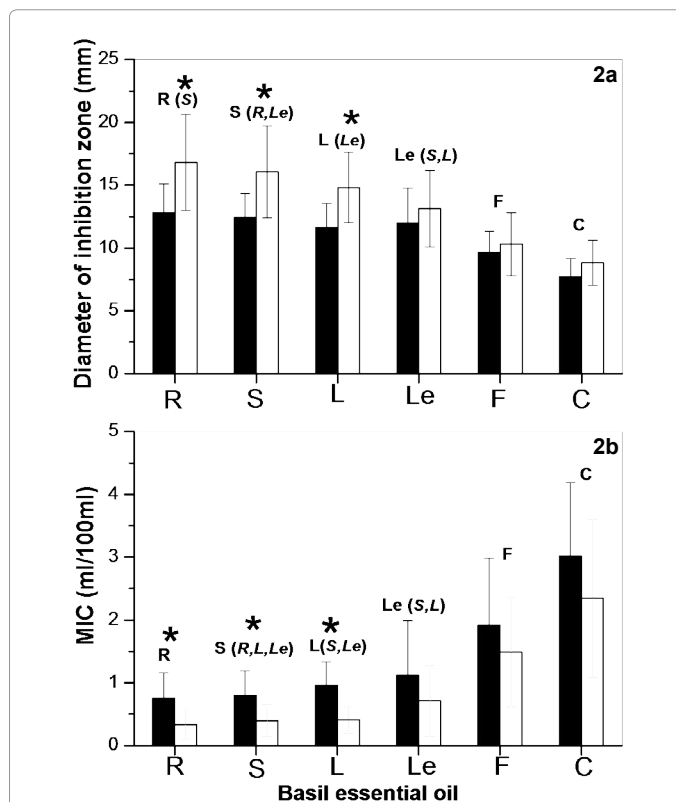


Figure 2: Antimicrobial activity of six basil essential oils. The average values (with standard deviations as error bars) of the diameter of inhibition zone (upper segment-Figure 2a) and minimum inhibitory concentration (MIC, lower segment-Figure 2b) observed after the application of the six basil essential oils under study [Red (R), Small-leaved (S), Large-leaved (L), Lettuce-leaved (Le), Lettuce-leaved Flower (F), Cinnamon (C)] on 17 Gram-negative (black columns) and 16 Gram-positive bacteria (white columns) are displayed. On the top of each column, the code name of the essential oil examined is accompanied in parenthesis by the code names of the other essential oils with which it shows similar antimicrobial activity. The asterisks denote the essential oils which display a statistically significant larger antimicrobial activity in Gram-positive relative to Gram-negative bacteria.

of the parameter I_f (derived from the fit of the experimental data to Equation 1) into y of Equation 2, we calculate the Trolox concentration $c=c_f$ (in μM) which is equivalent to the $x\%$ v/v concentration of EO employed during the test of its antioxidant activity. Finally, in order to account for the different EO concentrations (values of x) used in the experiments and express the antioxidant capacity of each EO in Trolox equivalents per ml of (non-diluted) EO (which is independent of the experimentally employed EO concentration, x) the following algebraic treatment is performed: when $x=0.1\%$ v/v it is deduced that 1 ml of EO is added to 1000 ml of DPPH control solution; thus for a calculated (via Equation 2) equivalent Trolox concentration equal to $c_f \mu\text{M}$ (or else $c_f \mu\text{mol}$ Trolox in 1000 ml DPPH control) it is concluded that the EO under study possesses an antioxidant capacity (referred to as Trolox equivalent antioxidant capacity, $\text{TEAC}_{\text{DPPH}}$) equal to $c_f \mu\text{mol}$ Trolox per ml of EO. In analogy, in the cases of $x=0.001\%$ v/v (0.01 ml of EO in 1000 ml) or 0.005% v/v (0.05 ml of EO in 1000 ml), the resulting values for $\text{TEAC}_{\text{DPPH}}$ equal $c_f/0.01$ and $c_f/0.05 \mu\text{mol}$ Trolox per ml of EO respectively. From the above presentation it is concluded that in general (for an EO experimentally employed concentration equal to $x\%$ v/v) the value of Trolox equivalent antioxidant capacity ($\text{TEAC}_{\text{DPPH}}$) expressed in μmol Trolox per ml of EO results by the division $c_f/10x$.

The theoretical fits of the experimental data points to equations 1 or 2 above were carried out using the program OriginPro 7.5 (OriginLab).

Statistical analysis

Statistical analysis was carried out using the SPSS v. 20 software. Due to the small sample sizes (<30 data points), nonparametric (or distribution-free) tests were employed. Thus, in order to compare two data sets, the Wilcoxon test or the Mann-Whitney test was employed for the case of paired samples and unpaired (independent) samples, respectively. In order to make multiple comparisons (ie. between three or more data sets), the Friedman test or the Kruskal-Wallis test was employed for the case of paired samples and unpaired (independent) samples, respectively. The non-parametric multiple comparison tests (Friedman, Kruskal-Wallis) do not provide the possibility to perform specific pairwise comparisons via post-hoc tests (eg. Bonferroni). In this case, in order to detect statistically significant differences between two specific data sets, multiple pairwise comparisons are performed via the use of either the Wilcoxon (paired samples) or the Mann-Whitney (independent samples) tests. All statistical tests were employed in order to detect statistically significant differences at the 95% significance level ($p<0.05$).

In order to test for differences in antimicrobial activity that depend on the bacterium group (Gram-positive vs. Gram-negative), the Mann-Whitney test was employed for each specific EO. The measurements of both experimental methods (DA and MIC) were examined. Thus, for each EO the following pairwise comparisons were made: a) DA data in Gram negative bacteria with DA data in Gram positive bacteria (Table 1, upper and lower part of a specific column) and b) MIC data in Gram negative bacteria with MIC data in Gram positive bacteria (Table 2, upper and lower part of a specific column).

In order to test for differences between the antimicrobial activities exhibited by the six different EOs, multiple pairwise comparisons were performed via the use of the Wilcoxon test (paired samples). More specifically, the following sets of comparisons were performed: a) comparisons between the antimicrobial activities of two different EOs exhibited in Gram-negative bacteria and probed with DA (two columns in upper part of Table 1, DA-Gram⁻), b) comparisons between the antimicrobial activities of two different EOs exhibited in Gram-

negative bacteria and probed with MIC (two columns in upper part of Table 2, MIC-Gram⁻), c) comparisons between the antimicrobial activities of two different EOs exhibited in Gram-positive bacteria and probed with DA (two columns in lower part of Table 1, DA-Gram⁺) and d) comparisons between the antimicrobial activities of two different EOs exhibited in Gram-positive bacteria and probed with MIC (two columns in lower part of Table 2, MIC-Gram⁺). The presentation of the results is done as follows: The code names of the essential oils are put in the order R – S – L – Le – F – C with the first (R) being the EO exhibiting the highest average value of diameter of inhibition zone (DA experiment) in Gram-positive bacteria and the last (C) corresponding to the EO which showed the lowest average value respectively. For each EO which showed no statistically significant difference ($p>0.05$) with another one, its code name (i.e. R, S, L, Le, F or C) is reported accompanied in parenthesis by the name(s) of the other EO(s) with which it shows statistically similar antimicrobial activity. Thus if an EO shows statistically significant differences in antimicrobial activity with all the other five EOs, it is reported solely with its code name, ie. with no accompanying parenthesis.

Results

Antimicrobial activity

The antimicrobial activity of the EOs was evaluated against seventeen Gram-negative bacteria and sixteen Gram-positive bacteria, by the disc diffusion assay (DA, Table 1). All bacteria were susceptible to all EOs tested, although the degree of inhibition varied among the EOs.

Strong inhibitory activity was exhibited by five of the basil EOs tested: Large-leaved basil EO inhibited strongly four bacterial strains, small-leaved basil EO inhibited strongly eight bacterial strains, red basil EO inhibited strongly eleven bacterial strains, lettuce-leaved basil EO inhibited strongly four, while the lettuce-leaved flower basil EO inhibited strongly one bacterial strain. No strong inhibition was observed when cinnamon basil EO was used.

Moderate inhibition was observed for bacterial strains for all EOs tested, although the number of strains inhibited varied among the EOs. Large-leaved basil EO inhibited moderately twenty eight bacterial strains, small-leaved basil EO inhibited moderately twenty five strains, lettuce-leaved basil EO inhibited moderately twenty three strains, red basil EO inhibited moderately twenty one strains, lettuce-leaved flower basil EO inhibited moderately seventeen strains and finally cinnamon basil EO inhibited moderately six bacterial strains.

Five of the EOs inhibited weakly the tested bacterial strains. Large-leaved and red basil EOs inhibited weakly one strain, lettuce-leaved basil EO inhibited weakly six strains, lettuce-leaved flower basil EO inhibited weakly fifteen strains and finally cinnamon basil EO inhibited weakly twenty seven strains.

The results from the measurement of minimum inhibitory concentration (MIC, Table 2) showed that the EOs have variable levels of inhibition. The lowest MIC value (0.039 ml/100 ml) was found for the small-leaved basil EO against *B. cereus* 10876 and the red basil EO against *M. luteus* 49732, *B. cereus* 14579 and *B. cereus* 10876. In contrast, the highest MIC (5 ml/100 ml) was obtained with lettuce-leaved flower EO against *S. tallahassee* 12002 and cinnamon basil EO against *S. schottmuelleri* 8759, *S. tallahassee* 12002, *S. typhimurium* 13311, *P. vulgaris* 5380, *S. epidermidis* 12228 and *S. saprophyticus* 3552.

Of the thirty three bacterial strains examined, the highest inhibitory

Microorganism	Basil type						Ery ^b
	R ^a	S ^a	L ^a	Le ^a	F ^a	C ^a	
Gram negative							
<i>S. arizonae</i> 13314	13.7 (0.6)	11.7 (0.6)	12.7 (0.6)	11.7 (0.6)	12.3 (0.6)	9 (1)	8.3 (0.6)
<i>S. typhimurium</i> 14028	12 (0)	10.7 (0.6)	10.3 (0.6)	12 (0)	11 (0)	7.3 (0.6)	Res
<i>S. schottmuelleri</i> 8759	11.3 (0.6)	10 (0)	10.3 (0.6)	10.7 (0.6)	10.3 (0.6)	6 (0)	7 (0)
<i>S. tallahassee</i> 12002	11 (1)	10 (0)	10.3 (0.6)	11.3 (0.6)	6 (0)	5.3 (0.6)	24.7 (0.6)
<i>S. enteritidis</i> 13076	15 (0)	13.7 (0.6)	14 (0)	15.3 (0.6)	11.3 (0.6)	7.7 (0.6)	Res
<i>S. typhimurium</i> 13311	11.3 (0.6)	10 (1)	10 (0)	9.7 (0.6)	8 (0)	6.3 (0.6)	25 (1)
<i>S. pullorum</i> 19945	17 (1)	16 (0)	15.3 (0.6)	17 (1)	10.7 (0.6)	8.7 (0.6)	Res
<i>K. pneumoniae</i> 13883	8.7 (0.6)	12.3 (0.6)	8.7 (0.6)	8 (0)	8 (0)	7 (0)	21.7 (0.6)
<i>K. pneumoniae</i> 10031	12.3 (0.6)	12 (0)	10.7 (0.6)	7.7 (0.6)	7.7 (0.6)	7 (0)	19.7 (0.6)
<i>E. coli</i> 11303	16 (0)	15 (0)	15.3 (0.6)	15.3 (0.6)	11.3 (0.6)	9.3 (0.6)	Res
<i>E. aerogenes</i> 35028	12.3 (0.6)	12.7 (0.6)	10.7 (0.6)	11.3 (0.6)	9 (0)	7.7 (0.6)	Res
<i>Proteus vulgaris</i> 5380	12.3 (1.1)	11.3 (0.6)	12.3 (0.6)	9.3 (0.6)	8.7 (0.6)	6 (0)	34.3 (0.6)
<i>P. vulgaris</i> 49132	9.7 (0.6)	11.7 (0.6)	10.3 (0.6)	9.3 (0.6)	8.3 (0.6)	7 (0)	19 (0)
<i>Shigella boydii</i> 9207	12.7 (0.6)	12 (0)	11.7 (0.6)	13.3 (0.6)	10.3 (0.6)	9.3 (0.6)	Res
<i>S. sonnei</i> 9290	15.3 (0.6)	14.7 (0.6)	11.7 (0.6)	14.7 (0.6)	10.6 (0.6)	9.3 (0.6)	7 (0)
<i>S. flexneri</i> 18022	16.3 (0.6)	15.3 (0.6)	13.7 (0.6)	16.3 (0.6)	12 (1)	10.3 (0.6)	9 (0)
<i>Y. enterocolitica</i> 23715	11.7 (0.6)	11 (1)	10 (1)	11.7 (0.6)	9 (1)	9 (0)	21.3 (0.6)
Gram positive							
<i>S. aureus</i> 12598	14 (0)	13.3 (0.6)	11.7 (0.6)	12.7 (0.6)	9.3 (0.6)	9.7 (0.6)	23.3 (0.6)
<i>S. aureus</i> 25923	15.3 (0.6)	13.7 (0.6)	13.3 (0.6)	15 (0)	9 (0)	8.7 (0.6)	24.3 (0.6)
<i>S. aureus</i> 12600	14 (0)	12.7 (0.6)	12.7 (0.6)	15.3 (0.6)	8.7 (0.6)	9 (0)	23 (1)
<i>S. epidermidis</i> 12228	13 (1)	12.7 (0.6)	12.7 (0.6)	8.7 (0.6)	7.3 (0.6)	6 (0)	22 (0)
<i>S. epidermidis</i> 14990	12.7 (0.6)	12.7 (0.6)	11.7 (0.6)	8.7 (0.6)	7 (0)	7.7 (0.6)	Res
<i>S. lentus</i> 700403	15.7 (0.6)	14.7 (0.6)	15.3 (0.6)	11.3 (0.6)	9.7 (0.6)	6.7 (0.6)	19.3 (0.6)
<i>S. hominis</i> 27844	17 (0)	15.7 (0.6)	14.7 (0.6)	10.7 (0.6)	10 (0)	7 (0)	23.7 (0.6)
<i>S. saprophyticus</i> 3552	13.7 (0.6)	13.3 (0.6)	12.3 (0.6)	10.3 (0.6)	8.7 (0.6)	6 (0)	20.3 (0.6)
<i>M. luteus</i> 533	20.7 (1.1)	20.7 (0.6)	15.7 (0.6)	14.7 (0.6)	13.7 (0.6)	12.3 (0.6)	34.3 (0.6)
<i>M. luteus</i> 49732	21.7 (0.6)	21.3 (1.1)	19.7 (0.6)	19.7 (0.6)	17.3 (0.6)	12.3 (0.6)	26.7 (0.6)
<i>M. luteus</i> 4698	16.3 (0.6)	15.7 (0.6)	15.3 (0.6)	12.7 (0.6)	12 (0)	10.3 (0.6)	19.7 (0.6)
<i>L. monocytogenes</i> 15313	15 (1)	13.7 (0.6)	14.7 (0.6)	13.7 (0.6)	12 (0)	9 (0)	9 (0)
<i>L. monocytogenes</i> 7644	13.3 (0.6)	11.7 (0.6)	11.7 (0.6)	11.7 (0.6)	9 (0)	9 (0)	25.7
<i>L. monocytogenes</i> 19111	19.3 (0.6)	18.7 (0.6)	14.7 (0.6)	18.3 (0.6)	10.3 (0.6)	9 (0)	29 (0)
<i>Bacillus cereus</i> 14579	22.3 (0.6)	20.7 (0.6)	18.3 (1.1)	11.7 (0.6)	10.3 (0.6)	8 (0)	16.7 (0.6)
<i>B. cereus</i> 10876	24.7 (0.6)	23.3 (0.6)	21.3 (0.6)	12.6 (0.6)	11.3 (0.6)	10.3 (0.6)	15 (0)

Mean values of three experimental trials are reported with standard deviation given in parenthesis. All cases of EOs showing increased antimicrobial activity relative to erythromycin are shown in bold;

^aBasil type: R: Red; S: Small-leaved; L: Large-leaved; Le: Lettuce-leaved; F: Lettuce-leaved Flower; C: Cinnamon

^bEry: erythromycin; Res: resistant to erythromycin.

Table 1: Disc diffusion assay (DA) results (diameter of zone of inhibition in mm).

activity in both assays (DA and MIC) was observed in two of them (*M. luteus* 533, *M. luteus* 49732) for all EOs examined. Furthermore, three more bacterial strains (*L. monocytogenes* 19111, *B. cereus* 14579 and *B. cereus* 10876) were strongly inhibited by the large-leaved, small-leaved and red basil EOs. All five bacteria belong to the Gram-positive group.

In the following paragraphs, the results of the statistical analyses conducted in order to probe differences between the exhibited antimicrobial activities depending on the bacterium group (Gram-positive vs. Gram-negative) and the basil variety are presented.

With regard to the dependence of the EO antimicrobial activity on the bacterium group the following results were obtained. When the Mann-Whitney test is conducted in the DA data, three out of the six examined EOs, namely L, S and R showed statistically significant ($p < 0.05$) increased antimicrobial activity against Gram-positive bacteria relative to Gram-negative ones. When the Mann-Whitney test is conducted in the MIC data, the same trend was shown for the same three out of the six examined EOs. Thus, taking into account the

results from the statistical analysis of both experimental methods (DA and MIC), we conclude that the experimental measurements provide strong evidence for the fact that the EOs of large-leaved, small-leaved and red basil display a larger (at 95% significance level) antimicrobial effect towards Gram-positive bacteria relative to Gram-negative ones. On the other hand, no such difference is evidenced for the lettuce-leaved, the lettuce-leaved flower and the cinnamon basil EOs.

With regard to differences between the antimicrobial activities exhibited by the six different EOs the following results were obtained. The Friedman test in both the DA and MIC data for both bacterium types showed that there exist statistically significant differences between the antimicrobial activities of the six basil EOs ($p < 0.05$). Subsequently, multiple pairwise Wilcoxon tests were performed and the outcomes for each of the four comparisons made (see Materials and Methods) are the following (see Materials and Methods for method of the results' presentation):

(a) DA-Gram: R(S) - S(R, Le) - L(Le) - Le(S, L) - F - C

Microorganism	Basil type						Ery ^b
	R ^a	S ^a	L ^a	Le ^a	F ^a	C ^a	
Gram negative							
<i>S. arizonae</i> 13314	0.63	0.63	0.63	0.63	0.63	2.5	2.5
<i>S. typhimurium</i> 14028	0.63	1.25	1.25	0.63	1.25	2.5	Res
<i>S. schottmuelleri</i> 8759	1.25	1.25	1.25	1.25	1.25	5.0	2.5
<i>S. tallahassee</i> 12002	1.25	1.25	1.25	1.25	5.0	5.0	0.039
<i>S. enteritidis</i> 13076	0.31	0.63	0.63	0.31	1.25	2.5	Res
<i>S. typhimurium</i> 13311	1.25	1.25	1.25	1.25	2.5	5.0	0.039
<i>S. pullorum</i> 19945	0.31	0.31	0.31	0.31	1.25	2.5	Res
<i>K. pneumoniae</i> 13883	1.25	0.63	1.25	2.5	2.5	2.5	0.078
<i>K. pneumoniae</i> 10031	0.63	0.63	1.25	2.5	2.5	2.5	0.078
<i>Escherichia coli</i> 11303	0.31	0.31	0.31	0.31	1.25	2.5	Res
<i>E. aerogenes</i> 35028	0.63	0.63	1.25	1.25	2.5	2.5	Res
<i>Proteus vulgaris</i> 5380	1.25	1.25	1.25	2.5	2.5	5.0	0.039
<i>P. vulgaris</i> 49132	1.25	0.63	1.25	2.5	2.5	2.5	0.078
<i>Shigella boydii</i> 9207	0.63	0.63	0.63	0.63	1.25	2.5	Res
<i>S. sonnei</i> 9290	0.31	0.31	0.63	0.31	1.25	2.5	2.5
<i>S. flexneri</i> 18022	0.31	0.31	0.63	0.31	0.63	1.25	2.5
<i>Y. enterocolitica</i> 23715	0.63	1.25	1.25	0.63	2.5	2.5	0.078
Gram positive							
<i>S. aureus</i> 12598	0.63	0.63	0.63	0.63	2.5	1.25	0.078
<i>S. aureus</i> 25923	0.31	0.63	0.63	0.31	2.5	2.5	0.078
<i>S. aureus</i> 12600	0.63	0.63	0.63	0.31	2.5	2.5	0.078
<i>S. epidermidis</i> 12228	0.63	0.63	0.63	2.5	2.5	5.0	0.078
<i>S. epidermidis</i> 14990	0.63	0.63	0.63	1.25	2.5	2.5	Res
<i>S. lentus</i> 700403	0.31	0.31	0.31	0.63	1.25	2.5	0.078
<i>S. hominis</i> 27844	0.31	0.31	0.31	0.63	1.25	2.5	0.078
<i>S. saprophyticus</i> 3552	0.31	0.63	0.63	1.25	1.25	5.0	0.078
<i>Micrococcus luteus</i> 533	0.078	0.078	0.31	0.31	0.31	0.63	0.039
<i>M. luteus</i> 49732	0.039	0.078	0.078	0.16	0.31	0.63	0.039
<i>M. luteus</i> 4698	0.31	0.31	0.31	0.63	0.63	1.25	0.078
<i>L. monocytogenes</i> 15313	0.31	0.63	0.31	0.63	0.63	2.5	2.5
<i>L. monocytogenes</i> 7644	0.63	0.63	0.63	0.63	1.25	2.5	0.039
<i>L. monocytogenes</i> 19111	0.16	0.16	0.31	0.31	1.25	2.5	0.039
<i>Bacillus cereus</i> 14579	0.039	0.078	0.16	0.63	2.5	2.5	0.078
<i>B. cereus</i> 10876	0.039	0.039	0.078	0.63	0.63	1.25	0.16

Mean values of three experimental trials are reported. All three trials gave the same MIC values in all measurements. All cases of EOs which show increased antimicrobial activity relative to Erythromycin are shown in bold.

^aBasil type: R: Red; S: Small-leaved; L: Large-leaved; Le: Lettuce-leaved; F: Lettuce-leaved Flower; C: Cinnamon

^bEry: erythromycin; Res: resistant to erythromycin

Table 2: Minimum inhibitory concentration (MIC) results (ml/100 ml).

(b) MIC-Gram⁻: R(S) - S(R, Le) - L(Le) - Le(S, L) - F - C

(c) DA-Gram⁺: R - S - L(Le) - Le(L) - F - C

(d) MIC-Gram⁺: R - S(L) - L(S) - Le - F - C

By combining the results of the Disc Assay experiment (a, c), we reach the following conclusion in relation with the differences between antimicrobial activities of the different EOs, irrespective of the bacterium group employed:

R(S) - S(R, Le) - L(Le) - Le(S, L) - F - C

These results are also shown graphically in the upper segment of Figure 2.

By combining the results of the MIC (b, d) experiment, we reach the following conclusion in relation with the differences between antimicrobial activities of the different EOs, irrespective of the bacterium type employed:

R - S(R, L, Le) - L(S, Le) - Le(S, L) - F - C

These results are also shown graphically in the lower segment of Figure 2.

Finally, by combining the results of all four comparisons (a-d) made above, we reach the following conclusion in relation to the possible differences between the antimicrobial activities of the different EOs, irrespective of both the bacterium type and the experimental method employed:

R(S) - S(R, L, Le) - L(S, Le) - Le(S, L) - F - C

The above combined result can also be expressed via the use of mathematical symbols as follows: R ≥ S ≈ L ≈ Le > F > C

Free radical scavenging activity

Figure 3 shows a characteristic DPPH absorbance (at 517 nm)

decay curve for each one of the six different basil EOs employed. The EO concentrations used (x% v/v) were the following: 0.001% v/v for large-leaved (L), small-leaved (S), lettuce-leaved (Le) and red (R) basil EOs, 0.005% for lettuce-leaved flower (F) basil EO and 0.1% for cinnamon (C) basil EO.

The theoretical fits to Equation 1 (see Materials and Methods) are shown superimposed on the experimental data points. In all six examined EOs, a double-exponential fit was needed in order to adequately account for the time dependence of the exhibited antioxidant activity. More specifically, all time decay curves possess a fast time component ($t_1=7-20$ minutes) and a slower one ($t_2=55-130$ minutes). The fast and slow time components accounted respectively for (67% ± 9%) and (33% ± 9%) of the total observed decay of the DPPH absorbance at 517 nm. The Trolox equivalent antioxidant capacity (TEAC_{DPPH}) values extracted from the fits (see Materials and Methods) are given in Table 3.

This approach for determining the DPPH antioxidant activity and subsequently expressing it in terms of Trolox equivalents presents two advantages: a) it is not dependent on the initial concentration of the DPPH radical stock solution and b) it provides the total antioxidant activity of the examined oil due to all types of antioxidants present in the sample (fast and slow acting, see below).

As it can be seen from the results of Table 3, the large-leaved (L) and small-leaved (S) basil EOs exhibit the highest antioxidant activity (against the DPPH radical) relative to the other four examined EOs.

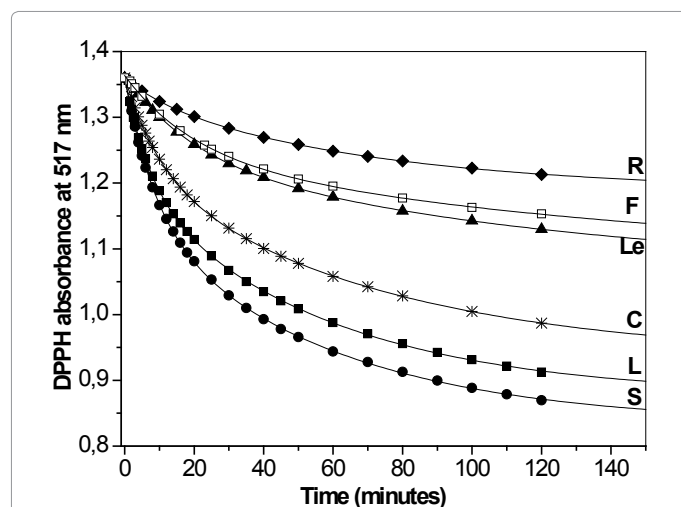


Figure 3: Free-radical scavenging capacity of basil essential oils. The time-decay curves of the DPPH absorbance at 517 nm of the following basil essential oils are displayed: Large-leaved (L, 0.001% v/v), Small-leaved (S, 0.001% v/v), Lettuce-leaved (Le, 0.001% v/v), Red (R, 0.001% v/v), Lettuce-leaved flower (F, 0.005% v/v) and Cinnamon (C, 0.1% v/v). Double exponential fits are shown superimposed.

Basil Variety	TEAC _{DPPH} (μmol Trolox/ml of essential oil)
Large-leaved (L)	1960 ± 50
Small-leaved (S)	2040 ± 140
Lettuce-leaved (Le)	1100 ± 90
Red (R)	645 ± 30
Lettuce-leaved flower (F)	195 ± 15
Cinnamon (C)	17 ± 1

Table 3: Trolox equivalent antioxidant capacity (TEAC_{DPPH}) of basil-based essential oils.

Performance of the Kruskal-Wallis statistical test showed statistically significant differences between the free radical scavenging activities of the six EOs ($p < 0.05$). Subsequently, multiple pairwise statistical comparisons via the Mann-Whitney test showed no statistically significant difference ($p > 0.05$) for the L-S pair and statistically significant differences for all the other independent pairs. Thus, it can be concluded that the antioxidant activity of the six examined basil EOs shows significant dependence on the basil variety as well as on plant part and it exhibits the following descending order: $L \approx S > Le > R > F > C$.

More specifically, the EO isolated from cinnamon basil (C) possesses the lowest free radical scavenging activity, while the EOs isolated from large-leaved (L) and small-leaved (S) basil showed the largest activities, namely ca. 120-fold higher relative to C. The EOs L and S are followed by the activities of Le, R and F which are ca 65-, 38- and 11-fold higher relative to C, respectively.

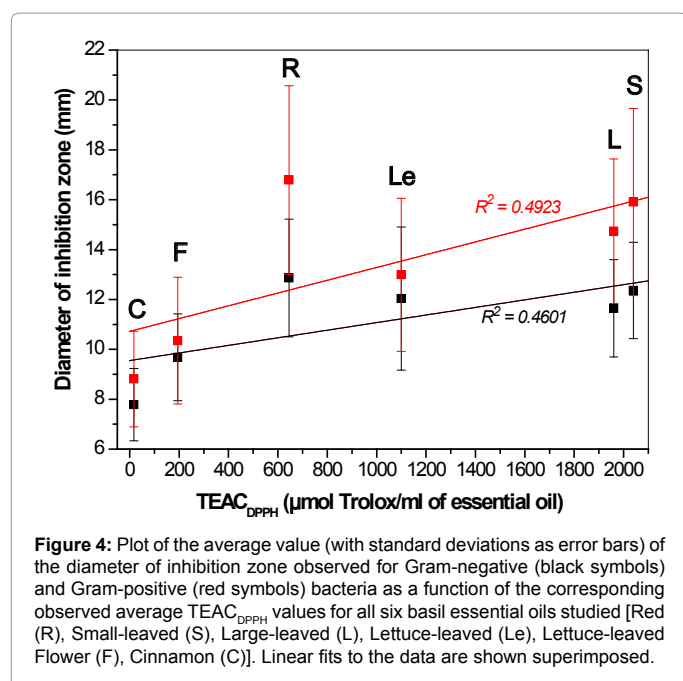
Correlation between antimicrobial and free radical scavenging activities

Finally, the possible correlation between antimicrobial and free radical scavenging activities was examined, which is an effect that has been observed previously in methanolic plant extracts [4]. For the six basil EOs examined in this work, it was noted that the EOs F and C which show the lowest antimicrobial activities, also showed the lowest antioxidant activities. However, the EO R was shown to be the most potent antimicrobial agent but a rather mediocre free radical scavenger (ca. 3-fold lower than the EOs L and S which are the most potent free radical scavengers). In Figure 4, the antimicrobial activity (average values of diameter of inhibition zone for Gram-negative and Gram-positive bacteria) of the EOs under study is depicted as a function of the corresponding free radical scavenging activity (TEAC_{DPPH} values). A qualitative examination of the data shows large scattering and a small (if any) correlation. In fact, an attempt to fit the two data sets to a linear relationship ($y = A \cdot x + B$) is also shown in Figure 4. The lines drawn correspond to the following equations: $y = 0.0015 \cdot x + 9.55$ (Gram-negative group, black line, $R^2 = 0.4601$) and $y = 0.0026 \cdot x + 10.7$ (Gram-positive group, red line, $R^2 = 0.4923$). Based on the poor quality of the fits, evidenced by their low R^2 values, it may be concluded that among the six basil EOs examined, there is no strong correlation between antimicrobial and free radical scavenging activities.

Discussion

In this work, a systematic effort was made in order to determine the antimicrobial and free-radical scavenging activities of EOs from five different basil varieties growing in Greece, namely red (R), small-leaved (S), large-leaved (L), lettuce-leaved (Le) and cinnamon (C) basil. In all varieties, the EO was isolated from the plant leaves while in one (lettuce-leaved) it was isolated from the plant flowers as well (F).

Three EOs, namely R, S and L showed statistically increased antimicrobial activity in Gram-positive bacteria relative to Gram-negative ones. There have been several other studies which have provided evidence for the increased sensitivity of Gram-positive bacteria to basil EO or extracts [4,15,21,23]. Possible explanations for this effect involve the significant differences in the outer membrane structure between the two types of bacteria [4]. However, as shown in our results and also pointed out by other researchers [4,23], the antimicrobial effect depends also on the specific tested organism, irrespective of Gram-group. In addition, the current study shows that the sensitivity of Gram-positive and Gram-negative bacteria to plant extracts and EOs depends also on the specific plant variety. In fact, in



contrast to what observed for the EOs isolated from the red, small-leaved and large-leaved basil varieties, the EOs from the remaining two basil varieties (lettuce-leaved and cinnamon) showed no statistically significant dependence of their antimicrobial activity on the Gram-group. Finally, it is important to note that the conclusions reached in the current work, are supported from a quite extensive experimental study based on the large number of tested microorganisms from both bacterium types and on the fact that it involved the exclusive use of reference bacterial strains (ATCC).

With regard to differences between the antimicrobial activities of the different EOs, the following major conclusions can be made:

a) The cinnamon (C) basil EO possesses statistically significant different antimicrobial activity from all the other basil EOs examined and more specifically the lowest one.

b) The lettuce-leaved Flower (F) basil EO possesses statistically significant different antimicrobial activity from all the other basil EOs examined and more specifically the next to the lowest.

c) The red (R) basil EO possesses statistically significant higher antimicrobial activity relative to the large-leaved (L) and lettuce-leaved (Le) basil EOs as well (ie in addition to the C and F EOs). On the other hand, the red (R) basil EO displays statistically similar antimicrobial activity with the EO of small-leaved (S) basil.

d) The antimicrobial activities of small-leaved (S), large-leaved (L) and lettuce-leaved (Le) basil EOs are statistically similar.

The above described dependence of basil EO antimicrobial activity on plant variety ($R \geq S \approx L \approx Le > C$) and plant part ($Le > F$) is indicative of differences in the EO chemical composition. This is also deduced by the observed (often dramatic) differences between the free radical scavenging activities of the six EOs.

With regard to the measurements of antioxidant activity, it should also be noted, that the necessity of using two time constants (one fast and one slower) in order to produce a satisfactory fit to the DPPH absorbance time decay curves, provides evidence that the observed

free radical scavenging activity is due to the action of more than one chemical compounds present in the EO under study. These compounds fall into two categories: the ones with a fast action and those with a slow action. This behavior has been observed before in the measurement of antioxidant activity of basil leaves extracts [30].

Conclusion

The antimicrobial activity of EO isolated from five different basil varieties growing in Greece was determined via measurements of diameter of inhibition zone and minimum inhibitory concentration. The antimicrobial effect was probed in 16 Gram-positive and 17 Gram-negative bacteria. A statistically significant increased susceptibility of the Gram-positive relative to the Gram-negative group was shown when using basil EOs of three plant varieties, namely red-leaved (R), small-leaved (S) and large-leaved (L). In addition, the observed basil EOs' antimicrobial activity showed statistically significant dependence on the plant variety and plant part (leaves vs. flowers).

Determination of the DPPH free radical scavenging activity of basil EOs showed a strong dependence on plant variety and plant part. The observed differences in antimicrobial and free radical scavenging activities are indicative of differences in the chemical composition of the corresponding essential oils.

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