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Biochemical activities of Iranian *Mentha piperita* L. and *Myrtus communis* L. essential oils

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Abstract

GC–MS analysis of essential oils of Iranian *Mentha piperita* and *Myrtus communis* extracted by hydrodistillation lead to identification of 26 and 32 compounds, respectively. The oils had good to excellent antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* with the oil of *M. piperita* being more active. The findings suggest feasibility of application of *M. piperita* oil in treatment of the infections caused by *C. albicans* and *E. coli*. *D*-values on exposure to *M. piperita* and *Myrtus communis* oils were (2.14 and 2.8 min), (1.4 and 12.8 min) and (4.3 and 8.6 min) for *E. coli*, *S. aureus* and *C. albicans* , respectively. The oils were screened for their possible antioxidant activities by two complementary test systems, namely DPPH free radical scavenging and β-carotene/linoleic acid systems. *M. piperirta* oil exerted greater antioxidant activity than that of *M. communis*. Phytochemical and phytobiological characteristics of these oils may lead to extraction and production of active compounds in single or combined forms with useful applications.

Keywords: *Mentha piperita*; *Myrtus communis*; Essential oil; *E. coli*; *S. aureus*; *C. albicans*; Antioxidant; Radical scavenging; Antimicrobial

1. Introduction

Development of microbial resistance to antibiotics is a global concern. Isolation of microbial agents less susceptible to regular antibiotics and recovery of increasing resistant isolates during antibacterial therapy is rising throughout the world which highlights the need for new principles. The use of essential oils as functional ingredients in foods, drinks, toiletries and cosmetics is gaining momentum, both for the growing interest of consumers in ingredients from natural sources and also because of increasing concern about potentially harmful synthetic additives (Reische et al., 1998). Within the wide range of the above-mentioned products, a common need is availability of natural extracts with a pleasant taste or fragrance combined with a preservative action, aimed at avoiding lipid deterioration, oxidation and spoilage by microorganisms. Until recently, essential oils have been studied mostly from their flavor and fragrance viewpoints only for flavoring foods, drinks and other goods. Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey et al., 2001). Many authors, in fact, have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties by essential oils (Gianni et al., 2005). Lipid peroxidation is a complex process occurring in aerobic cells and reflects the interaction between molecular oxygen and polyunsaturated fatty acids. Radicals are known to take part in lipid peroxidation, which

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causes food deterioration, aging organisms and cancer promotion (Ashok and Ali, 1999; Cerruti, 1994). Antioxidants act as radical-scavengers, and inhibit lipid peroxidation and other free radical-mediated processes: therefore, they are able to protect the human body from several diseases attributed to the reactions of radicals (Nizamuddin, 1987; Takao et al., 1994). Use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects (Cornwell et al., 1998; Faure et al., 1990; Feher and Pronai, 1993), making attractive the search for antioxidant and scavenger natural compounds. The present study explores such attractive biological features of *Mentha piperita* L. and *Myrtus communis* L. from Iran.

2. Results and discussion

2.1. Chemical composition of the essential oils

The results obtained by GC and GC–MS analysis of the essential oils of *M. piperita* and *M. communis* are presented in Tables 1 and 2, respectively. Twenty six and thirty two compounds were identified in the essential oils of *M. piperita* and *M. communis*, respectively. As a result of GC and GC–MS analyses, *M. piperita* contained α-terpinene (19.7%), isomenthone (10.3%), *trans*-carveol (14.5%), pipertitinone oxide (19.3%), and β-caryophyllene (7.6%) as the major compounds. α-Pinene (29.1%), limonene (21.5%), 1,8-cineole (17.9%), and linalool (10.4%) were the major compounds of *M. communis* oil.

Table 1
Chemical composition of *Mentha piperita* L. essential oil

No.	Compound	RI	%
1	Tricyclene	911	1.2
2	Camphene	947	1.2
3	Sabinene	952	1.8
4	β-Pinene	963	2.5
5	α-Terpinene	1007	19.7
6	1,8-Cineole	1020	0.17
7	Limonene	1031	0.7
8	γ-Terpinene	1037	0.6
9	Terpinolene	1062	0.08
10	*p*-Cymene	1072	0.15
11	Menthone	1127	0.8
12	Isomenthone	1137	10.3
13	Menthol	1149	3.6
14	Menthopuran	1156	2.3
15	γ-Terpineole	1159	2.7
16	Isomenthol	1179	0.2
17	*trans*-Carveol	1208	14.5
18	Carvone	1214	0.46
19	Piperitone oxide	1280	1.9
20	*p*-Menthon-3,8-diol	1301	0.2
21	Pipertitinone oxide	1330	19.3
22	*p*-Menth-1-en-9-ol	1363	0.16
23	Geranyl acetate	1369	0.12
24	(*E*)-β-Bourbonene	1374	1.2
25	α-Garjunene	1395	0.14
26	β-Caryophyllene	1407	7.6

Table 2
Chemical composition of *Myrtus communis* essential oil

No.	Compound	RI	%
1	Isobutyl isobutyrate	892	0.8
2	α-Thujene	922	0.3
3	α-Pinene	931	29.1
4	Sabinene	971	0.6
5	Myrcene	981	0.2
6	δ-3-Carene	998	0.2
7	*p*-Cymene	1013	0.3
8	Limonene	1025	21.5
9	1,8-Cineole	1028	17.9
10	(*E*)-Ocimene	1038	0.1
11	γ-Terpinene	1051	0.6
12	Terpinolene	1082	0.3
13	Linalool	1089	10.4
14	α-Campholenal	1122	0.03
15	*trans*-Pinocarveole	1130	0.07
16	δ-Terpineole	1154	0.09
17	Terpinene-4-ol	1169	0.5
18	α-Terpineole	1180	3.17
19	*trans*-Carveole	1213	0.4
20	*cis*-Carveole	1217	0.07
21	Geraniol	1242	1.1
22	Linalyl acetate	1248	4.8
23	Methyl geranate	1310	0.2
24	α-Terpinyl acetate	1342	1.3
25	Neryl acetate	1351	0.09
26	Methyl eugenol	1369	1.6
27	β-Caryophyllene	1430	0.2
28	α-Humulene	1463	0.2
29	Spathulenol	1562	0..07
30	Caryophylleneb epoxide	1586	0.1
31	Humulene epoxide II	1608	0.08
32	Acetocyclohexane dione (2)	1704	0.4

2.2. Antimicrobial activity

As can be seen in Table 3, the essential oils were found to have good to excellent antimicrobial activities against all microorganisms tested. The essential oil of *M. piperita* was found to be more active than the essential oil of *M. communis*. *M. piperita* oil was active at higher dilutions against microbial strains employed. *Candida albicans* was the most sensitive micro organism to both oils with higher vulnerability to *M. piperita* oil. *Escherichia coli* was the second vulnerable micro organism to the essential oil of *M. piperita*. The findings suggest feasibility of application of *M. piperita* oil in treatment of the infections caused by *C. albicans* and *E. coli*. The findings are in agreement with those of Mimica-Dukic et al. (2003) who reported low MIC (4 μl/ml) of *M. piperita* oil for *E. coli*. The *M. piperita* oil used in our study was fungicidal at 1/4 dilution (2 μl/ml) against *C. albicans* while Mimica-Dukic et al. (2003) reported to be 8 μl/ml and Hammer et al. (1999) reported cidal activity of *M. piperita* oil at 0.25% (v/v) equivalent to 25 μl/ml for *E. coli* and *C. albicans* and 12 μl/ml for *Staphylococcus aureus*. *M. piperita* oil was also reported to be cidal to *C. albicans* at 500 ppm (Tampieri et al., 2005). *M. piperita* antimicrobial activity was recorded only to *S. aureus* and not to *E. coli* (Aridogan et al., 2002). The oil used in the

Table 3
Determination of MIC, MLC and disk diffusion assay of the essential oils

Essential oil	Microorganism	Oil dilution				Mean inhibition zone (mm)
		1/8 (1 μl/ml)	1/4 (2 μl/ml)	1/2 (4 μl/ml)	1 (8 μl/ml)	
Mentha piperita	*E. coli*	+++	+	S	S	31.33
	S. aureus	++	+	S	S	11.67
	C. albicans	+	S	S	S	90
Myrtus communis	*E. coli*	+++	++	+	S	13
	S. aureus	+++	++	+	S	10
	C. albicans	++	+	S	S	21.33

+, MIC (minimal inhibitory concentration).
S, MLC (minimal lethal concentration).
++, low to medium growth.
+++, medium to good growth.

present study was lethal to both *S. aureus* and *E. coli*. These difference could be due to a difference in chemical composition of the oils. Menthol has been reported to be responsible for the antimicrobial activity of *M. piperita* (Iscan et al., 2002). Our results suggest that this does not seem to be the sole agent responsible for antimicrobial property of *M. piperita*. The present study reveals higher antimicrobial property of *M. piperita* essential oil with menthol concentration as low as 3.6%. Hence it can be deduced that other chemical compounds present in the oil have contributed to the antimicrobial property of *M. piperita* essential oil. In the case of *M. communis*, results from the disc diffusion method, and determination of minimal inhibitory and lethal concentrations (MIC and MLC), indicate that, *C. albicans* is the most sensitive microorganism with the lowest MLC value of 1/2 oil dilution (4 μl/ml). *E. coli* and *S. aureus* were almost equally affected by myrtle oil. This oil contained 1,8-cineole (17.9%) which was reported to impart microbicidal effect on *E. coli*, *S.aureus* and *C. albicans* (Cox et al., 2001).

2.3. Decimal reduction time (*D*-value)

Figs. 1 and 2 show decimal reduction time of *E. coli*, *S. aureus* and *C. albicans* brought about by the essential oils of *M. piperita* and *M. communis*, respectively, after exposure to the MLC levels of the oils. *D*-values on exposure to *M. piperirta* L. and *M. communis* L. oils were (2.14 and 2.8 min), (1.4 and 12.8 min) and (4.3 and 8.6 min) for *E. coli*, *S. aureus* and *C. albicans*, respectively. These values suggest variations in vulnerability of different micro organisms to different volatile oils. Duration of time required for complete lethal effects of the oils can be calculated from these figures for practical and applied purposes. Figs. 3–5 clearly show that oil concentration does not directly determine the size of microbial growth inhibition zone. It also implies that greater inhibition zone does not necessarily

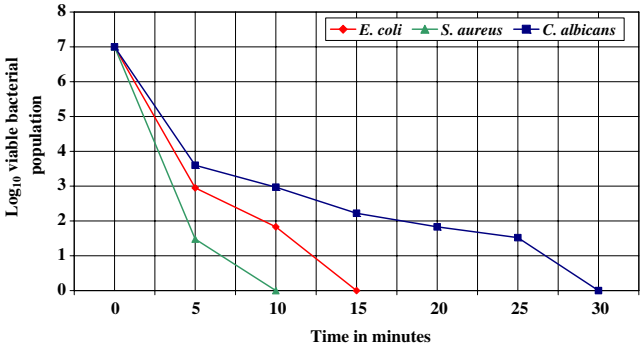

Fig. 1. Decimal reduction time (*D*-value) of microbial strains exposed to the MBC levels of *Mentha piperita* essential oil.

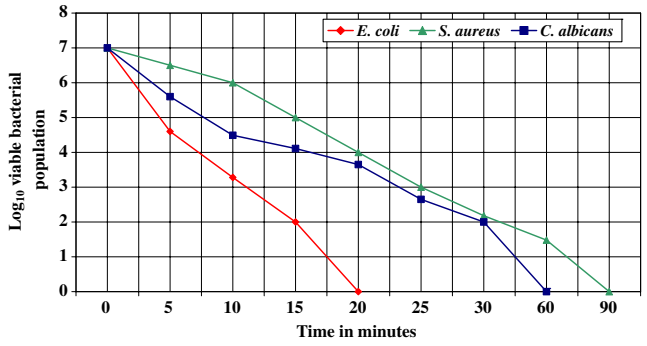

Fig. 2. Decimal reduction time (*D*-value) of microbial strains exposed to the MBC levels of *Myrtus communis* essential oil.

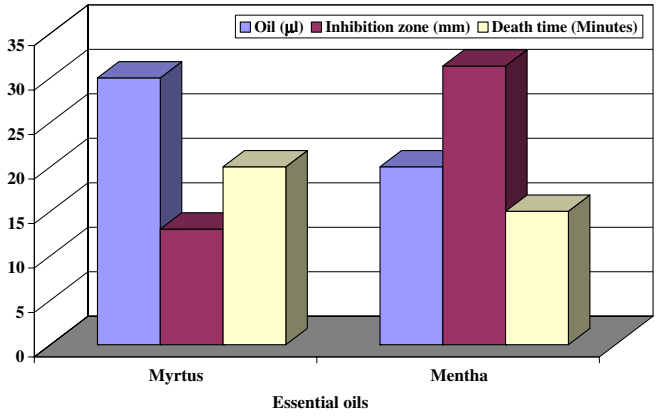

Fig. 3. Effect of MLC levels of the essential oils on the zone of inhibition and complete elimination of *E. coli*.

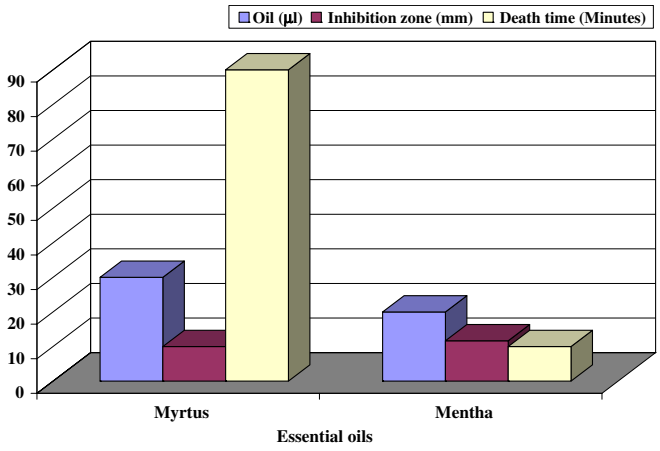

Fig. 4. Effect of MLC levels of the essential oils on the zone of inhibition and complete elimination of *S. aureus*.

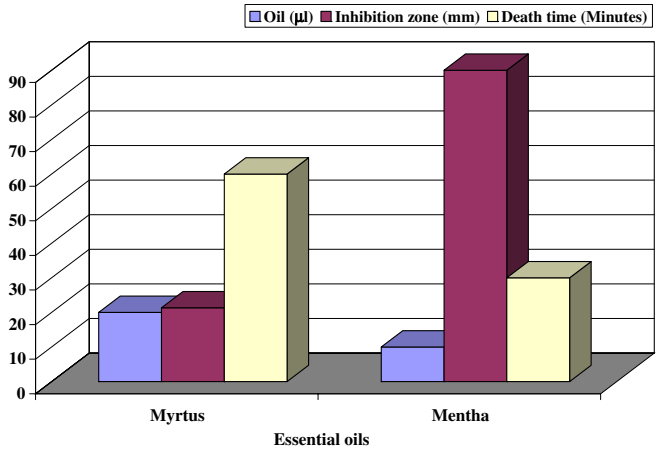

Fig. 5. Effect of MLC levels of the essential oils on the zone of inhibition and complete elimination of *C. albicans*.

reduce *D*-value or the duration wherein lethal effects take place.

2.4. Free radical scavenging capacity of the oils

The DPPH radical-scavenging activities of the essential oils and of references are shown in Fig. 6. *M. piperita* and *M. communis* essential oils notably reduced the concentration of DPPH free radical, with an efficacy lower than that of reference oil *Thymus X-porlock* (69.29% inhibition) and slightly lower than that of trolox. The performance of the *M. piperita* oil was better than that of *M. communis*. Oils with higher monoterpenic abundance were reported to be almost ineffective. This result is in agreement with the poor performance given by the oils with similar patterns and by single monoterpenic hydrocarbons (Ruberto and Baratta, 2000). *M. piperita* oil was reported to reduce DPPH to 50% (Mimica-Dukic et al., 2003). The reason for lower activity of our *M. piperita* oil than that of Mimica-Dukic et al. (2003) is higher dilution (five-fold) of the oil used in the present study confirming dose dependence of radical scavenging capacity of the oils. The most powerful scavenging compounds were reported to be monoterpene ketones (menthone and isomenthone) and 1,8-cineole (Mimica-Dukic et al., 2003). Our results are not in agreement with this report. The amount of menthone, isomenthone and 1,8-cineole in *M. piperita* used in the present study were 0.8%, 10.3% and 0.17%, respectively (Table 1). On the other hand our myrtle oil contained 17.9% of 1,8-cineole with a lower free radical scavenging activity of 3.53%.

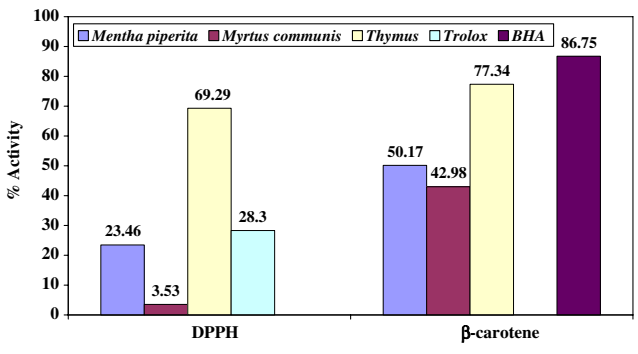

Fig. 6. Free radical scavenging and antioxidant activities of essential oils in comparison with those of the references.

2.5. β-Carotene-linoleic acid assay

The lipid peroxidation inhibitory activity of the essential oils were assessed by the β-carotene bleaching test (Fig. 6). Results of the reference oil (*Thymus X-porlock*) were almost consistent with data obtained from the DPPH test. Overall results were better than those provided by the radical-scavenging activity. The difference is probably as a consequence of a higher specificity of the assay for lipophilic compounds.

These properties are also very much needed by the food industry in order to find possible alternatives to synthetic preservatives (namely BHT, phenolics). Oxidative stress is involved in the pathogenesis of numerous diseases. Nevertheless, no optimal natural antioxidant has been found for therapeutics, therefore polyphenol antioxidants have been looked for in myrtle leaves, a plant that in folk medicine has been used as anti-inflammatory drug. These results suggest that the myrtle extracts have a potent antioxidant activity mainly due to the presence of galloyl derivatives (Romani et al., 2004).

3. Conclusion

In conclusion, *M. piperita* and *M. communis* essential oils, gave interesting results, being one of the promising extracts in terms of both antimicrobial activity and ability to neutralize free radicals and prevent unsaturated fatty acid oxidation. The results presented here may also contribute to knowledge of the antioxidative and antimicrobial potentials of these species reported elsewhere. Phytochemical and

phytobiological characteristics of these oils may lead to extraction and production of active compounds in single or combined forms with useful applications.

4. Experimental

4.1. General

The major equipments used were Clevenger, GC (9-A-Shimadzu), GC/MS (Varian-3400), Shimadzu UV-2501PC spectrophotometer. Microbial culture media (Merck), other chemicals were of analytical grade.

4.2. Plant materials

M. piperita L. was collected from Damavand district of Tehran-Iran during June/July 2005 and *M. communis* L. was from Dezful city of Iran during October/November 2005.

4.3. Oil extraction and analysis

The plant materials were steam distilled for 90 min in full glass apparatus. The oils were isolated using a Clevenger-type apparatus. The extraction was carried out for 2 h after a 4-h maceration in 500 ml of water. The oils were stored in dark glass bottles in a freezer until they were used. 1/2, 1/4 and 1/8 dilutions of the oils were made with dimethylsulphoxide (DMSO). These dilutions were used in antibacterial analysis. Undiluted oil was taken as dilution 1. GC analysis was performed by GC (9-A-Shimadzu) gas chromatograph equipped with a flame ionization detector. Quantitation was carried out on Euro Chrom 2000 from KNAUER by area normalization method. The analysis was carried out using a DB-5 fused-silica column (30 m × 0.25 mm, film thickness 0.25 μm) using a temperature program of 40–250 °C at a rate of 4 °C/min, injector temperature 250 °C, detector temperature 265 °C, carrier gas: helium (99.99%). The GC/MS unit consisted of Varian-3400 gas chromatograph coupled to a Saturn II ion trap detector. The column was same as of the GC under the same conditions stated above. The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention indices with those of authentic compounds or with literature data.

4.4. Microbial strain and growth media

E. coli (ATCC 25922), *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 5027) were employed in the study. Nutrient agar was used to culture *E. coli* and *S. aureus* at 37 °C. Sabouraud dextrose agar was used to culture *C. albicans* at 30 °C. Bacterial suspensions were made in Brain Heart Infusion (BHI) broth to a concentration of approximately 10^8 cfu/ml using standard routine spectrophometrical methods. Suspensions of *C. albicans* were made in Sabouraud dextrose broth. Subsequent dilutions were made from the above suspensions, which were then used in the tests.

4.5. Oil dilution solvent

Microbial strains were streaked on Mueller Hinton agar plates using sterile cotton swabs. 5 μl of dimethylsulphoxide (DMSO) loaded on sterile blank disks were placed on the agar plates and were incubated at appropriate temperatures for 24–48 h. There was no antimicrobial activity on the plates and hence DMSO was selected as a safe diluting agent for the oil. The oils were filter sterilized using a 0.45 μm membrane filter. 5 μl from each sterile oil dilutions was added to sterile blank discs. The solvent also served as control.

4.6. Disc diffusion method

The agar disc diffusion method was employed for the determination of antimicrobial activities of the essential oils in question. Briefly, 0.1 ml from 10^8 cfu/ml microbial suspension was spread on the Mueller Hinton Agar (MHA) plates. Sterile filter paper discs (6 mm in diameter) were impregnated with 5μl of the oil and were placed on the inoculated plates. These plates, after remaining at 4 °C for 2 h, were incubated for 24 h at 37 °C (for bacteria) and for 48 h at 30 °C (for yeast). The diameters of the inhibition zones were measured in millimeters. All tests were performed in triplicate.

4.7. Determination of minimum inhibitory (MIC) and lethal (MLC) concentrations

In order to assure sterility of the oils, geometric dilutions, ranging from 0.036 to 72.0 mg/ml of the essential oil, were prepared in a 96-well microtitre plate, including one control (BHI + Tween 80) and one sterility control (BHI + Tween 80 + test oil). Plates were incubated under normal atmospheric conditions, at 37 °C for 24 h. The contamination of the oils could be detected by microbial growth indicated by the presence of a white "pellet" on the well bottom. All tests were performed after confirming the sterility of the oils. Brain Heart Infusion (BHI) broth supplemented with Tween 80 detergent (final concentration of 0.5% (v/v) was used for all tests with the exception of *C. albicans* (Sabouraud dextrose broth with Tween 80)). Test strains were suspended in BHI broth (for bacteria) and Sabouraud dextrose broth (for yeast) to give a final density of 10^7 cfu/ml and these were confirmed by viable counts. The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) were assessed according to our modified procedure (Rasooli and Mirmostafa, 2003). MIC was determined by a broth dilution method in test tubes as follows: 40 μl from each of various dilutions

of the oils was added to 5 ml of broth tubes containing 10^7 cfu/ml of live cells. The tubes were then incubated on an incubator shaker. The highest dilution (lowest concentration), showing no visible growth, was regarded as MIC. 0.1 ml of the cell suspensions from the tubes showing no growth were subcultured on nutrient agar plates for bacteria and on Sabouraud dextrose agar plates for yeast to determine if the inhibition was reversible or permanent. Each experiment was performed in triplicate. MLC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

4.8. Microbicidal kinetics of the oils

Forty microlitres of the each oil at the dilution determined by MLC was added to each 5 ml of relevant broth tubes containing microbial suspension of 10^7 cfu/ml and were then incubated in an incubator shaker. 0.1 ml samples were taken after 5, 10, 15, 20, 25, 30, 45, and 90 min. The samples were immediately washed with 2 ml of sterile phosphate buffer pH 7.0, centrifuged at 10,000 rpm/1 min, resuspended in the buffer and were then spread cultured on appropriate agar plates for 24 h at 37 °C for bacteria and at 30 °C for the yeast. Phosphate buffer was used as diluent. Microbicidal experiments were performed three times. Microbial colonies were counted from triplicates after incubation period and the mean total number of viable cells per ml was calculated.

4.9. Calculation of decimal reduction time (D-value)

The mean total number of viable bacteria or yeast from bactericidal kinetics experiments at each time interval was converted to $\log_{10}$ viable cells using routine mathematical formulae. The time required to decrease the microbial population in a sample by 90% on the basis of logarithmic value of one was regarded as *D*-value.

4.10. Radical scavenging capacity of the oils

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,20-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits and Bucar, 2000., Cuendet et al., 1997). Fifty microlitres of 1:5 concentrations of the essential oils in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. Trolox (1 mM) (Sigma–Aldrich), a stable antioxidant, was used as a synthetic reference. The essential oil from *Thymus x-porlock* was used as a natural reference. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent ($I\%$) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Tests were carried out in triplicate.

4.11. β-Carotene–linoleic acid assay

Antioxidant activity of essential oils was determined using β-carotene bleaching test (Taga et al., 1984). Approximately 10 mg of β-carotene (type I synthetic, Sigma–Aldrich) was dissolved in 10 ml of chloroform. The carotene–chloroform solution, 0.2 ml, was pipetted into a boiling flask containing 20 mg linoleic acid (Sigma–Aldrich) and 200 mg Tween 40 (Sigma–Aldrich). Chloroform was removed using a rotary evaporator at 40 °C for 5 min and, to the residue, 50 ml of distilled water were added, slowly with vigorous agitation, to form an emulsion. Five millilitres of the emulsion were added to a tube containing 0.2 ml of essential oils solution prepared according to Choi et al. (2000) and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50 °C and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60 min period. Control samples contained 10 μl of water instead of essential oils. Butylated hydroxy anisole (BHA; Sigma–Aldrich), a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as inhibition percentage with reference to the control after 60 min incubation using the following equation: $AA = 100(\text{DR}_\text{C} - \text{DR}_\text{S})/\text{DR}_\text{C}$, where AA is the antioxidant activity; DR_C is the degradation rate of the control $= [\ln(a/b)/60]$; DR_S is the degradation rate in the presence of the sample $= [\ln(a/b)/60]$; a is the absorbance at time 0; b is the absorbance at 60 min.

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