Antimicrobial Activity of Mint Essential Oils

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The essential oils obtained from two mint species, *Mentha pulegium* and *Mentha spicata*, exhibited antimicrobial properties against eight strains of Gram-positive and Gram-negative bacteria. The essential oils of these mint species at high concentration (1/100 dilution) were extremely bactericidal, whereas lower concentrations (1/1000) caused a dose-dependent decrease in bacterial growth rates. The main *p*-menthane components of the tested essential oils showed a variable degree of antimicrobial activity not only between different bacterial strains but also between different strains of the same bacteria.

Keywords: *Mentha pulegium; M. spicata; essential oils; p-menthane compounds; antimicrobial activity*

INTRODUCTION

Herbs and spices have traditionally been used to extend the shelf life of foods and in folk medicine. Recently the interest in the biological activities of plant extracts has been rekindled and has been the subject of intense scientific investigation. Essential oils derived from many plants are known to possess biological activity against prokaryotic (Deans and Ritchie, 1987; Janssen et al., 1987) and eukaryotic organisms (Chaturvedi and Tripathi, 1989; Thompson, 1989; Konstantopoulou et al., 1992).

The existence of different chemotypes, based on qualitative differences within a taxon, is a common feature in most *Mentha* species and hybrids (Kokkini, 1991). Summarizing research published to date, it can be concluded that the main monoterpenes which characterize the essential oil composition of the different species and hybrids are either acyclic or cyclic C-2 (such as carvone, dihydrocarvone) or C-3 (such as menthone, pulegone) substituted compounds (Kokkini, 1992).

Previous studies concerning the antimicrobial properties of mint essential oils (Maiti et al., 1985; Janssen et al., 1986) yielded results that are difficult to reconcile. This is mainly due to the great variation found in the chemical composition of mint essential oils and to a lesser extent to differences in the experimental techniques applied (Janssen et al., 1987).

This lack of consistent results coupled with the importance of *Mentha* essential oils to the cosmetics and food industries led us into a comparative study of their antimicrobial activities with regard to two parameters: (i) the main *p*-menthane components of the source mint plant and (ii) the experimental techniques applied.

MATERIALS AND METHODS

Plant Material. GC and GC–MS Analyses of Essential Oils. Wild-growing fully flowered *Mentha pulegium* L. plants were collected from three localities of Greece: the island of Paros (M1), Mt. Vermio (M2), and Mt. Pangaio (M3). *Mentha spicata* L. plants (M4) were cultivated near the town of Edhessa (northern Greece). Voucher specimens are kept in the Herbarium of the Laboratory of Systematic Botany and Phytogeography, University of Thessaloniki.

The air-dried plant material (aerial parts) was pulverized and the essential oils isolated after hydrodistillation (*European Pharmacopoeia*, 1975) for 2 h. The four essential oils were chromatographed using a Shimadzu GC-14A gas chromatograph equipped with a Supelcowax 10 (Supelco, Bellefonte, PA) capillary column (60 m × 0.25 mm i.d.) and a flame ionization detector (FID). The carrier gas was helium, and the linear gas velocity was 20.4 cm/s. The injection and FID temperatures were 240 °C. Column temperature was initially 70 °C and was then gradually increased at a rate of 4 °C/min to 220 °C. Gas chromatography–mass spectroscopy (GC–MS) analyses were conducted using a Shimadzu GC–MS QP2000 system equipped with a capillary column under the same GC conditions (see above). For GC–MS detection a quadropolar system was used with ionization energy of 70 eV. The essential oil components were identified by comparing their relative retention times (RRT) and mass spectra (MS) with those of authentic samples and literature citations (Stenhagen et al., 1974; Masada, 1976; Jennings and Shibamoto, 1980) as well as a computerized MS data bank.

Antimicrobial Assay (Disk Diffusion Assay). The following reference strains of bacteria, purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland) were used as test organisms in all antimicrobial assays: *Escherichia coli* (NCIMB 8879 and NCIMB 12210), *Pseudomonas aeruginosa* (NCIMB 12469), *Salmonella typhimurium* (NCIMB 10248), *Staphylococcus aureus* (NCIMB 9518 and NCIMB 8625), *Rhizobium leguminosarum* (NCIMB 11478), and *Bacillus subtilis* (NCIMB 3610). Bacteria were grown either in nutrient broth or in nutrient agar and incubated at 37 °C (*E. coli, P. aeruginosa, S. typhimurium*), at 30 °C (*S. aureus, B. subtilis*), or at 25 °C (*R. leguminosarum*). These particular strains are standard reference strains that are routinely used for the evaluation of antimicrobial compounds.

Filter paper disks (Whatman No. 1, 5 mm diameter) containing 5 μL of the particular essential oil or isolated compound (authentic samples, Aldrich Chemical Co., Milwaukee, WI) were applied to the surface of agar plates that were previously seeded by spreading of 0.2 mL of bacterial overnight culture. The plates were incubated overnight at the appropriate temperature (see above), and the diameter of the resulting zone of inhibition was measured in millimeters. The results indicated in Tables 2 and 3 and in the text represent the net

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Table 1. Quantitative Composition (Percent Area Percentage Analysis) of *M. pulegium* and *M. spicata* Essential Oils with Respect to the *p*-Menthane Components Tested for Antimicrobial Activity

	M. pulegium			*M. spicata*
	M1	M2	M3	M4
C-3 substitution components				
menthlol	t[a]	0.4	0.1	nd[b]
isomenthol	0.2	1.3	0.1	nd
neomenthol	0.6	0.6	t	nd
menthone	1.7	1.0	10.3	nd
isomenthone	0.2	4.5	77.5	nd
pulegone	50.6	44.7	1.0	nd
isopulegol	0.2	0.4	0.3	nd
piperitone	13.4	1.9	0.3	nd
C-2 substitution components				
carvone	nd	nd	nd	33.4
trans-dihydrocarvone	nd	nd	nd	0.3
cis-dihydrocarvone	nd	nd	nd	11.4
trans-carveol	nd	nd	nd	0.9
cis-carveol	nd	nd	nd	3.5
dihydrocarveol	nd	nd	nd	34.8
neoiso-dihydrocarveol	nd	nd	nd	1.8
essential oil yields (mL/100 g of dry wt of plant tissue)	1.7	1.6	2.0	0.3

[a] Trace, <0.05%. [b] Not detected.

zone of inhibition after subtraction of the diameter (5 mm) of the paper disk.

Determination of Bacterial Cell Growth. Well-isolated, single bacterial colonies from overnight plates were transferred into nutrient broth and grown overnight at the appropriate temperature (see above). Tubes of nutrient broth containing various concentrations (as indicated in the figure legends) of essential oils were then inoculated with appropriate aliquots of these cultures so that their optical densities (OD_{600}) were equal. The growth of each culture was monitored in two ways, either by measuring its optical density at 600 nm (OD_{600}) at 30 min intervals for a total period of 8 h (total counts) or by plating on nutrient agar at specific time intervals suitably diluted aliquots of the culture (viable counts).

Determination of Minimum Inhibitory Concentration (MIC). The appropriate amount of the essential oil dissolved in 95% ethanol was added to nutrient broth. Doubling serial dilutions were prepared from this broth and were then inoculated with bacteria to a final concentration of 10^6 cells/ml. In each case control broths containing solvent (95% ethanol) or ampicillin (20 μg/mL) were also prepared. After overnight incubation at the appropriate temperature, the MIC was read. MIC was defined as the lowest concentration of essential oil that totally inhibited bacterial growth.

RESULTS AND DISCUSSION

Qualitative and quantitative analysis of the four *M. pulegium* (M1, M2, M3) and *M. spicata* (M4) essential oils showed that their bulk (54.8–89.6% of the total oil) consisted of cyclic *p*-menthane compounds. As can be seen from Table 1, all three *M. pulegium* oils (M1, M2, M3) were rich in C-3 substituted compounds, although their individual levels varied. In particular, the concentrations of the three ketones, isomenthone (0.2–77.5% of the total oil), pulegone (1.0–50.6%), and piperitone (0.3–13.4%), varied greatly among the three oils. The *M. spicata* essential oil (M4) was mainly characterized by high concentrations of carvone and dihydrocarveol.

The antimicrobial properties of the four *Mentha* essential oils were initially evaluated by disk diffusion assay against a panel of eight bacterial strains. The results are presented in Table 2 and show that the essential oils exhibited a variable degree of antimicrobial activity against all of the bacterial strains tested

Table 2. Antimicrobial Activity of Mint Essential Oils, Obtained by Disk Diffusion Assay[a]

	M. pulegium			*M. spicata*
bacteria	M1	M2	M3	M4
E. coli (12210)	10	6	8	8
E. coli (8879)	11	9	14	11
S. typhimurium (10248)	11	8	8	7
P. aeruginosa (12469)	4	1	–[b]	–
R. leguminosarum (11478)	5	6	7	5
S. aureus (9518)	16	12	11	7
S. aureus (8625)	10	10	12	11
B. subtilis (3610)	13	13	10	6

[a] The diameter (mm) of the inhibitory zone represents the mean of three independent experiments. (The diameter of the paper disk, 5 mm, is not included.) [b] No activity.

with the exception of *P. aeruginosa*, which appeared to be sensitive only to the pulegone-rich *M. pulegium* essential oils (M1, M2). In general, the three *M. pulegium* essential oils showed slightly higher levels of antimicrobial activity compared to that of *M. spicata*. It should be pointed out that in the disk diffusion assay one cannot differentiate between the bactericidal and bacteriostatic effects exerted by the essential oils.

The second set of experiments was designed to examine the effect of the essential oils on the bacteria in relation to cumulative time of exposure and oil concentration. From the panel of bacteria used in the initial disk diffusion experiments, we selected one Gram-positive, *S. aureus* (NCIMB 8625), and one Gram-negative, *E. coli* (NCIMB 12210), bacterial strain. The choice of these two bacterial strains was based on their relevance to public health and their almost uniform sensitivity to the M3 and M4 essential oils. The results are presented in Figure 1 and show that treatment of both bacteria with the two essential oils resulted in dose-dependent inhibition of their growth. Both essential oils at 1/100 dilution were highly bactericidal against both bacteria. In the case of *E. coli* both oils at 1/100 dilution killed all of the bacteria within 30 min after exposure, whereas in the case of *S. aureus* total kill was attained within 3 h and was slightly faster for the *M. pulegium* oil (M3). At 1/1000 dilution the essential oils continued to show high bactericidal activity against *E. coli*; total kill was obtained within 30 min after exposure. Only slight bactericidal activity was seen against *S. aureus*. At 1/5000 dilution neither essential oil showed bactericidal activity against either bacterium and caused only a small decrease in growth rate during the 3 h incubation period for which the cultures were monitored. Comparable results were obtained with two additional Gram-negative bacteria (*S. typhimurium* NCIMB 10248, and *E. coli*, NCIMB 8879).

The decreased growth rates observed at the 1/5000 dilution of both essential oils prompted us to examine their effect on bacterial growth rates at high dilutions (1/5000, 1/10000, and 1/50000) and over longer periods of exposure. Since at these dilutions neither essential oil exhibits appreciable bactericidal action, the bacterial growth kinetics were monitored spectrophotometrically at 600 nm. The results, presented in Figure 2, showed that both M3 and M4 oils at dilutions of up to 1/10000 caused a small dose-dependent decrease in the growth rate of both bacteria. In the case of *S. aureus* the decrease in growth rate either remained almost constant (*M. pulegium*, M3) or increased (*M. spicata*, M4) during the course of the incubation period. In the case of *E. coli* the net bacterial growth reached a plateau approximately 5 h after addition of the essential oils.

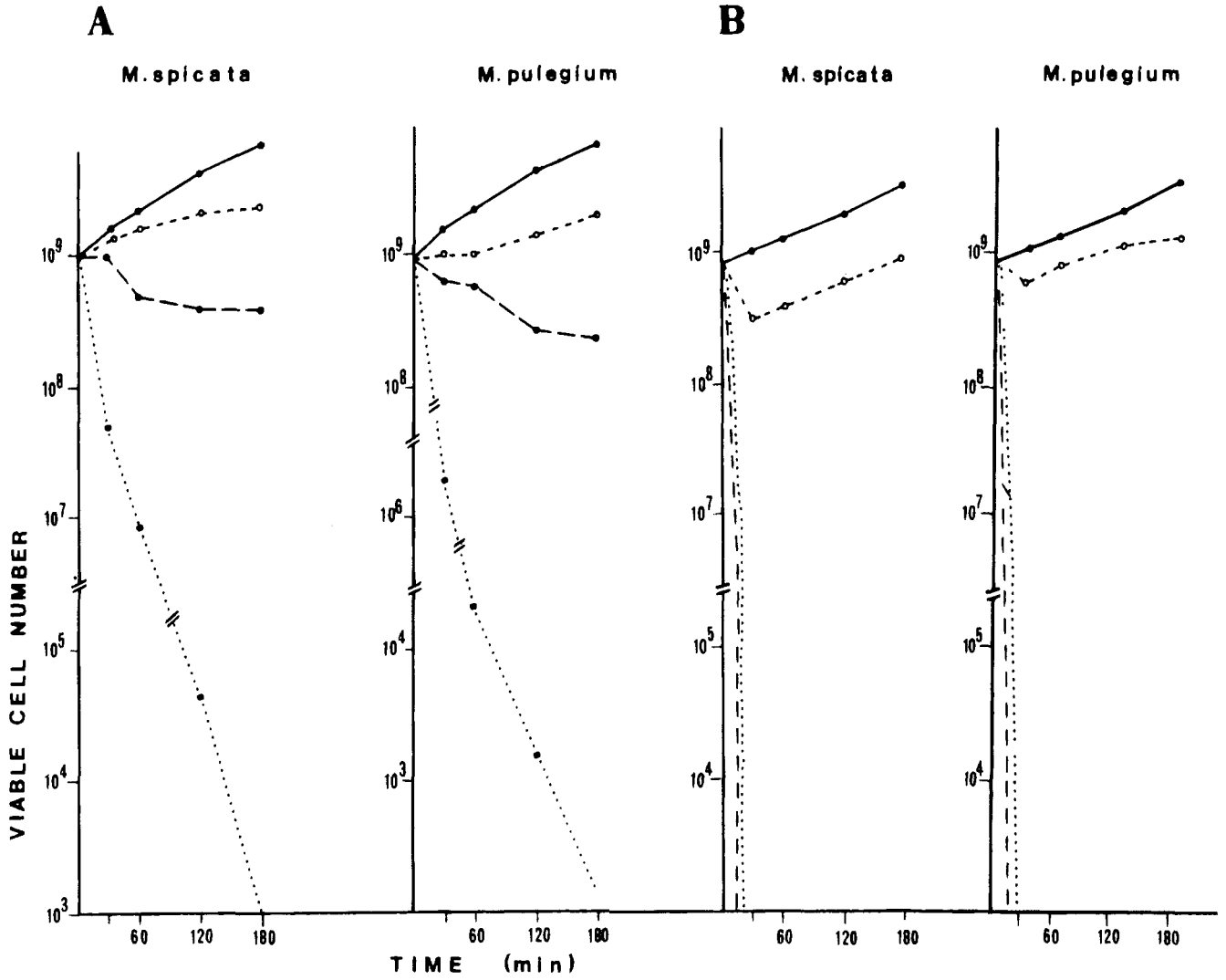

Figure 1. Time- and concentration-dependent effect of essential oils obtained from *M. pulegium* (M3) and *M. spicata* (M4) on the viability of *S. aureus* (NCIMB 8625) (panel A) and *E. coli* (NCIMB 12210) (panel B). Equal aliquots of overnight bacterial cultures were inoculated in equal amounts of nutrient broth supplemented with or without various concentrations of the two essential oils. At specific time intervals, suitably diluted aliquots of all cultures were plated on nutrient agar and the viable cell number was counted. These experiments were repeated independently three times and yielded essentially the same results. Panels A and B: control (—), 1/100 (···), 1/1000 (– –), 1/5000 (- - -) of essential oils.

Finally, the minimum concentration that completely inhibits bacterial growth (MIC) over a 24 h incubation period was determined. Both M3 and M4 essential oils at 1/32 dilution completely inhibited the growth of 10^6 cells of *E. coli* (NCIMB 12210) or *S. aureus* (NCIMB 8625).

In an effort to evaluate the individual contributions of the main components of the mint essential oils to the antimicrobial properties, we evaluated the activity of the individual main compounds by disk diffusion assay, using authentic commercially available preparations. The results presented in Table 3 show that all compounds exhibited a variable degree of antimicrobial activity with the exception of isomenthol, whose activity was negligible. Pulegone showed the highest activity against *S. typhimurium*, while it was inactive against *S. aureus* strain NCIMB 9518. On the contrary, isomenthone showed the highest activity against the same strain of *S. aureus* (NCIMB 9518), while it was inactive against *S. typhimurium*. Notably, *P. aeruginosa* showed resistance to all tested compounds with the exception of pulegone, isopulegol, and piperitone. These compounds were not detected in *M. spicata* essential oil and represent a very low percentage (1.6% of the total oil) in *M. pulegium* M3 oil, consistent with the lack of activity of both oils against *P. aeruginosa* observed in the disk diffusion assay experiments (see Table 2). On the contrary, mint essential oils rich in pulegone, such as M1 or M2, showed increased activity against *P. aeruginosa*. Recently, Panizzi et al. (1993) reported that of four essential oils tested in their studies, *P. aeruginosa* was sensitive only to *Calamintha nepeta* oil, the only pulegone-containing oil of the four. Furthermore, the data presented here indicate that, in addition to pulegone, piperitone may also be important for activity since the difference observed in the susceptibility of *P. aeruginosa* to the M1 and M2 oils (4 vs 1 mm diameter of inhibition zone, respectively) is possibly due to the greater relative concentration of piperitone in M1 than in M2 oil (13.4 vs 1.9%, respectively). This is also supported by the data of Panizzi et al. (1993) since *C. nepeta* oil was also the only essential oil of the four tested that contained piperitone.

The *p*-menthane compounds showed differential degrees of activity even between different strains of the same bacterium; e.g., *E. coli* strain NCIMB 8879 was almost twice as susceptible as *E. coli* strain NCIMB 12210 to dihydrocarveol and carveol (representing in

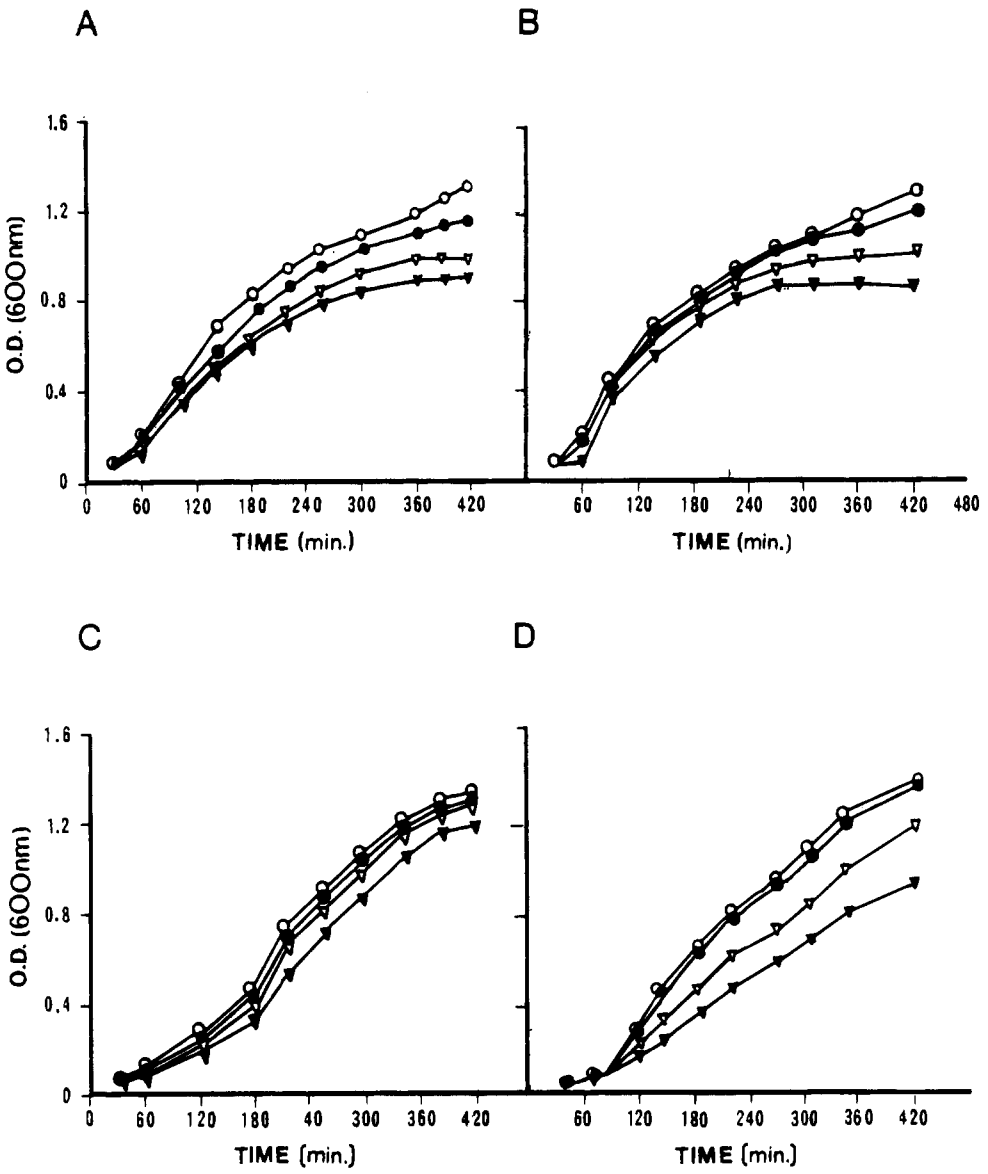

Figure 2. Time- and concentration-dependent effect of essential oils obtained from *M. pulegium* (M3) (panels A and C) or *M. spicata* (M4) (panels B and D) on rates of growth of *E. coli* (NCIMB 12210; panels A and B) and *S. aureus* (NCIMB 8625; panels C and D). Equal aliquots of overnight bacterial cultures were inoculated in equal amounts of nutrient broth supplemented with or without various concentrations of essential oils, and cell growth was monitored spectrophotometrically at 600 nm at specific time intervals. These experiments were repeated independently three times and yielded essentially the same results. Control (○), 1/50000 (●), 1/10000 (▽), 1/5000 (▼) of essential oils.

Table 3. Antimicrobial Activity of the *p*-Menthane Compounds Dominant in Mint Essential Oils

	bacterial strains according to their NCIMB number							
	12210	8879	10248	12469	11478	9518	8625	3610
carvone	4	–[b]	6	–	4	5	1	5
dihydrocarvone[c]	3.5	4	2	–	4	4	2	3
carveol	7	13	5	–	7	2	2	9
dihydrocarveol	7	13	8	–	6	4	5	6
menthol	–	1	–	–	3	2	2	2
isomenthol	–	–	–	–	1	–	–	–
neomenthol	4	5	5	–	5	3	3	7
menthone	3	3	3	–	4	6	6	4
isomenthone	1	3	–	–	3	4	2	2
pulegone	2	5	9	6	5	–	4	5
isopulegol	4	5	10	10	11	2	3	5
piperitone	5	7	5	5	7	4	–	7

[a] Five microliter samples of each compound were spotted (undiluted) on paper disks as indicated under Materials and Methods. The diameter (mm) of the inhibition zone is the mean of three independent experiments. (The diameter of paper disk, 5 mm, is not included.) [b] No activity. [c] Consisting of *cis*- and *trans*-isomers.

total 40% of the *M. spicata* M4 essential oil). These differences may explain the higher antimicrobial activity of the *M. spicata* essential oil (M4) against *E. coli* strain NCIMB 8879 than against *E. coli* strain NCIMB

12210. Similarly, the *M. pulegium* essential oils (M1, M2, M3) showed higher antimicrobial activity against *E. coli* strain NCIMB 8879 than against *E. coli* strain NCIMB 12210. This difference is in agreement with the observed higher susceptibility of *E. coli* strain 8879 to all of the main compounds of *M. pulegium* essential oil.

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