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Chemical Composition and Antibacterial Activity of Selected Essential Oils and Some of Their Main compounds

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The chemical composition of essential oils of cabreuva (*Myrocarpus fastigiatus* Allemao, Fabaceae) from Brazil, cedarwood (*Juniperus ashei*, Cupressaceae) from Texas, Juniper berries (*Juniperus communis* L., Cupressaceae) and myrrh (*Commiphora myrrha* (Nees) Engl., Burseraceae) were analyzed using GC/FID and GC/MS. The antimicrobial activity of these essential oils and some of their main compounds were tested against eleven different strains of Gram-positive and Gram-negative bacteria by using agar diffusion and agar serial dilution methods. Animal and plant pathogens, food poisoning and spoilage bacteria were selected. The volatile oils exhibited considerable inhibitory effects against all tested organisms, except *Pseudomonas*, using both test methods. Higher activity was observed against Gram-positive strains in comparison with Gram-negative bacteria. Cabreuva oil from Brazil showed similar results, but in comparison with the other oils tested, only when higher concentrations of oil were used.

Keywords: Cabreuva oil, *Myrocarpus fastigiatus*, Cedarwood oil, *Juniperus asheii,* Juniper berries oil, *Juniperus communis,* myrrh oil*, Commiphora myrrha,* chemical composition, antibacterial activity.

Natural compounds obtained from various plants and plant materials have been extensively used in medicine, aromatherapy, food and the cosmetic industries. Essential oils of plant origin are extensively reported as potent antimicrobials and antifungals [1-6]. These oils are further known for their antioxidant effects [7] and possess additional auxiliary values for health as they express anti-inflammatory, antiviral [8,9], anti-tumor and anticarcinogenic effects [10].

The primary uses of essential oils for medicinal, food and perfumery applications vary. Cedarwood oil is mainly employed in perfumery for dry nuances in citrus and woody compositions [11,12], cabreuva oil in perfumery due to its pleasant smell [12], and juniper berry oil for flavoring herbal infusions and drinks, in perfumery for creating fresh and dry effects, and as an aroma ingredient in alcoholic beverages of the gin-type [12]. Myrrh oil is produced from air-dried gum of *Commiphora myrrha* shrubs that are predominantly found in northeastern Africa and Arabia. The application of myrrh essential oil (obtained by steam distillation of the gum) is not limited to perfumery, but it can also be used to provide an aromatic bitter taste [12] in flavorings. The aim of this paper was first, to investigate the chemical composition of the essential oils of cabreuva from Brazil, juniper berries, cedarwood from Texas, and myrrh, and secondly, to assess the antimicrobial activity of these oils against several clinical and food-isolated Gram-positive (*Bacillus cereus*, *Staphylococcus aureus* and *S. epidermis*) and Gram-negative bacteria (*Citrobacter diversus*, *Escherichia coli*, *Pseudomonas aeruginosas, P. fluorescens,* and *Salmonella abony*). Furthermore, some

Table 1a: Chemical composition of cabreuva essential oil from Brazil.

RI †	RI ¥	Compound	Area§ [%]
1457	1794	Geranyl acetone	0.4
1469	-	*epi*-β-Santalene	0.4
1486	1692	β-Santalene	0.4
1516	1708	β–Bisabolene	1.4
1523	-	(*Z*)-γ-Bisabolene	0.6
1532	1735	δ-Cadinene	1.0
1539	1993	(*Z*)-Nerolidol	0.1
1549	1753	(*E*)-γ-Bisabolene	0.6
1552	1884	α-Calacorene	0.1
1578	2027	(*E*)-Nerolidol	77.0
1649	-	δ-Cadinol	1.3
1661	2201	α-Cadinol	1.3
1672	2181	Bulnesol	0.8
1685	2189	*epi*-α-Bisabolol	1.4
1687	2191	α-Bisabolol	1.6
1697	2201	(*Z,E*)-Farnesol	1.1
1722	2331	(*E,E*)-Farnesol	2.1
		Total	91.6

† SE-54 column, ¥ Carbowax column, § %-peak area using GC/FID.

Table 1b: Chemical composition of myrrh essential oil.

RI †	RI ¥	Compound	Area§ [%]
938	-	*α*-Pinene	0.1
954	-	Camphene	0.1
1047	-	(*E*)-Ocimene	0.1
1343	1459	δ-Elemene	0.8
1383	1484	α-Copaene	0.2
1394	1510	β-Bourbonene	0.7
1400	1576	β-Elemene	4.0
1428	1583	β-Caryophyllene	0.3
1439	1621	γ-Elemene	0.6
1441	1571	(*E*)-α-Bergamotene	0.3
1462	1650	α-Humulene	0.2
1489	1690	Germacrene D	1.0
1508	1846	Curcerene	24.9
1529	1735	γ-Cadinene	0.5
1637	2091	Furanoeudesm-1,3-diene*	33.0
1642	2094	Furanoeudesm-1,3-diene*	9.7
1645	2193	τ-Cadinol	0.7
1675	2007	Elemol acetate	1.4
1696	2188	Germacrone	0.6
		Total	79.2

*unidentified isomers.

of the major constituents of these essential oils were tested against the same microbes to evaluate their influence with respect to the total composition. Using GC/FID and GC/MS the chemical compositions of these four essential oils were analyzed (Tables 1a-d).

The essential oil of cabreuva (*Myrocarpus fastigiatus* Allemao, Fabaceae) was dominated by 77% of (*E*)-nerolidol, a sesquiterpene alcohol with an extremely pleasant olfactory profile [12]. Olfactory evaluation of cabreuva oil features tender balsamic, dusty floral somewhat herbal and woody inspirations.

The essential oil of myrrh has a soft resinous, woody, warm balsamic, and oriental herbaceous scent; the main compounds identified were the two furanoeudesm-1,3-diene isomers (42.7%), curcerene (24.9%) and β-elemene (4.0%). This result is in good accordance with studies carried out in Iran by Morteza-Semnani *et al*, [13], in which curcerene was identified as the main compound.

Table 1c: Chemical composition of cedarwood oil from Texas.

RI †	RI ¥	Compound	Area§ [%]
940	1013	α-Pinene	0.5
1034	-	Limonene	0.3
1153	1501	Camphor	0.
1429	1560	α-Cedrene	8.6
1437	1587	β-Cedrene	3.4
1452	1615	Thujopsene	38.4
1464	1626	α-Himachalene	0.2
1478	1655	α-Acoradiene	0.3
1480	1661	β-Acoradiene	0.6
1492	1675	γ-Himachalene	1.2
-	1684	β-Chamigrene	0.1
1515	1691	β-Himachalene	3.5
1519	1717	α-Chamigrene	1.6
1521	-	Cuparene	1.8
1557	-	(*E*)-*N*erolidol	0.3
1621	2090	Cedrol	28.6
1642	2100	α-Acorenol	1.6
1646	2129	β-Acorenol	0.7
		Total	91.7

Table 1d: Chemical composition of juniper berries oil.

RI †	RI ¥	Compound	Area§ [%]
929	1000	Tricyclene	0.1
932	-	α-Thujene	0.7
943	1018	α-Pinene	35.4
954	1045	α-Fenchene	0.1
956	1053	Camphene	0.5
960	-	Thuja-2,4(10)-diene	0.1
981	1110	Sabinene	7.6
986	1097	β-Pinene	3.3
990	1120	*p*-Mentha-2,8-diene	0.3
997	1150	Myrcene	15.3
1010	1156	*p*-Mentha-1(7),8-diene	0.7
1016	1135	δ-*3*-Carene	0.1
1022	1165	α-Terpinene	0.5
1030	1251	*p*-Cymene	2.1
1035	1187	Limonene	7.3
1049	-	(*E*)-Ocimene	0.1
1063	1230	γ-Terpinene	1.8
1095	1267	Terpinolene	1.2
1104	-	Linalool	0.2
1148	-	(*E*)-Pinocarveol	0.1
1187	-	Terpinen-4-ol	2.4
1199	-	α-Terpineol	0.2
1227	-	Fenchyl acetate	0.1
1293	-	Bornyl acetate	0.2
1355	-	Terpinyl acetate	0.1
1359	1443	α-Cubebene	0.5
1387	1478	α-Copaene	0.5
1402	1569	β-Elemene	0.6
1433	1579	β-Caryophyllene	4.2
1443	1599	Thujopsene	0.3
1462	-	(*E*)-β-Farnesene	0.3
1466	1645	α-Humulene	1.2
1493	1685	Germacrene D	1.8
1524	-	γ-Cadinene	0.4
1533	1730	δ-Cadinene	1.5
		Total	91.9

Cedarwood oil from Texas was evaluated as harsh, woody, typical cedar with a somewhat earthy and burnt odor; chemical evaluation by GC/MS identified thujopsene (38.4%), cedrol (28.6%), α-cedrene (8.6%) and β-himachalene (3.5%) as main compounds.

Essential oil of juniper berries was assessed as fresh, terpenous, green, herbaceous and reminiscent of the alcoholic beverage "Gin"; the main compounds identified were α-pinene (35.4%), myrcene (15.3%),

limonene (7.3%), β-caryophyllene (4.2%) and β-pinene (3.3%).

The results of the antimicrobial tests performed by disc diffusion are reported in Table 2. An inhibition zone ranging from 0-9 mm was seen as insignificant antimicrobial activity, whereas an inhibition zone from 10 to 15 mm was interpreted as mildly active, from 15 to 19 mm as moderately active, and from 20 mm and beyond as good. Gram-positive bacteria were inhibited by all essential oils in the test except cabreuva oil, Gram-negative bacteria were not significantly inhibited by juniper berries and cedar wood oil, whereas myrrh oil showed moderate activity towards *E. coli* and *Salmonella* species. None of the essential oils tested showed antimicrobial activity towards *Pseudomonas* species in terms of inhibition zones (IZs).

Single compounds identified as major components of the tested essential oils were α-bisabolol, (*E*)- and (*Z*)-nerolidol, and (*E,E*)-farnesol (cabreuva oil), cedrol and thujopsene (cedarwood oil), and sabinene (juniper berries oil).

The antimicrobial activity of single compounds using IZs can be summarized as follows: All single compounds produced an IZ greater than 10 mm towards Gram-positive bacteria, especially (*E*)- and (*Z*)-nerolidol, which produced the largest IZs, whereas these compounds were not active against *Pseudomonas* species in the test. IZs of the above-mentioned compounds towards Gram-negative bacteria were insignificant, except for (*E*)- and (*Z*)-nerolidol against *E. coli*, and thujopsene against *Salmonella abony* clinical isolate.

Results from serial broth dilution assays resulting in minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) are presented in Tables 3 and 4. The MICs defined as substance concentration [%] to prevent the growth of bacteria within a period of time in *in vitro* assay obtained from the agar dilution method are shown in Table 3.

Overall reduction of Gram-negative bacteria was accomplished by concentrations of 0.2 to 0.4% of myrrh, cedarwood and juniper berries oil, except against *P aeruginosa* and *P. fluorescens* with MICs of 0.8% (v/v). The MIC for cabreuva oil towards Gram-positive bacteria in the test was 0.4% (v/v) and 0.8% (v/v) towards Gram-negative bacteria.

General reduction of microbial growth was achieved by α-bisabolol, and (*E*)- and (*Z*)-nerolidol at concentrations of 0.1% towards Gram-positive bacteria, cedrol and thujopsene at 0.2% towards the same Gram-positive bacteria, and 0.4% for (*E,E*)-farnesol and sabinene.

To reduce growth of Gram-negative bacteria, except *Pseudomonas*, α-bisabolol, (*E*)- and (*Z*)-nerolidol are needed in concentrations of 0.2%, cedrol, thujopsene and sabinene at 0.4%, except sabinene towards *C. diversus*, and for cedrol, thujopsene and sabinene against *Pseudomonas* species, 0.8% was necessary.

The Minimal Bactericidal Concentration (MBC) is defined as the lowest concentration of an agent that shows no evidence of microbial growth in the determination assay. Gram-positive bacteria were inhibited at concentrations of or below 0.2% (v/v) by myrhh oil. The MBCs for cedarwood and juniper berries oil were 0.4% (v/v) towards Gram-positive bacteria and also 0.4% for juniper berries oil against *Citrobacter diversus* and *E.coli*. The MBC for *Pseudomonas* species was 0.8% for myrrh, cedarwood, and juniper berries oil, and 1% for cabreuva oil.

The MBCs of the pure substances tested were 0.2% for α-bisabolol, (*E*)- and (*Z*)-nerolidol towards Gram-positive bacteria, 0.4% for cedrol, thujopsene and sabinene against Gram-positive bacteria, and likewise for α-bisabolol, (*E*)- and (*Z*)-nerolidol to kill initial colonies of *C. diversus, E. coli* and *Salmonella* species. To obtain comparable activity in all other cases a minimum concentration of 0.8% was required, especially against *Pseudomonas* species.

Table 2: Antibacterial activity of test essential oils and main compounds - Inhibitory zones [mm].

Test microorganism	CO	CW	JB	MY	α-bisa-bolol	cedrol	(*E,E*)-farnesol	(*E*)-ne-rolidol	(*Z*)-ne-rolidol	sabi-nene	thujop-sene
B. cereus (ATCC 11778)	0	13.5	12.5	13.5	12.5	13	11	11	11.5	12	10.5
S. aureus (ATCC 6538)	0	18.5	16	22	18	15	13	23	24	18	14
S. aureus (food spoilage isolate)	0	19	16.5	24	17.5	16	14	21	21.5	18	16.5
S. epidermidis (clinical isolate)	0	16.5	14.5	21	16.5	14	14	22	22	16.5	15
C. diversus (clinical isolate)	0	0	7	6*	7	0	9	0	0	6	0
E. coli (ATCC 8739)	0	8	8	10	9	6	8	10	11	8	6
P. aeruginosa (ATCC 9627)	0	0	0	0	0	0	0	0	0	0	0
P. aeruginosa (clinical isolate)	0	0	0	0	0	0	0	0	0	0	0
P. fluorescens (food spoilage isolate)	0	0	0	0	0	0	0	0	0	0	0
S. abony (ATCC 6017)	0	6	9	9	9	7	0	0	0	0	9
S. abony (clinical isolate)	0	6	9	10	9	7	0	0	0	0	10

*IZ below 9 mm can be seen as insignificant. CO= cabreuva oil, CW= cedarwood oil, JB= juniper berries oil, MY= myrrh oil

Table 3: Antibacterial activity of several essential oils and main compounds - Minimal Inhibitory Concentration [%].

Test microorganism	CO	CW	JB	MY	α-bisa-bolol	cedrol	(*E,E*)-farnesol	(*E*)-ne-rolidol	(*Z*)-ne-rolidol	sabi-nene	thujop-sene
B. cereus (ATCC 11778)	0.4	0.2	0.2	0.1	0.1	0.2	0.4	0.1	0.1	0.4	0.2
S. aureus (ATCC 6538)	0.4	0.2	0.2	0.1	0.1	0.2	0.4	0.1	0.1	0.4	0.2
S. aureus (food spoilage isolate)	0.4	0.2	0.2	0.1	0.1	0.2	0.4	0.1	0.1	0.4	0.2
S. epidermidis(clinical isolate)	0.4	0.2	0.2	0.1	0.1	0.2	0.4	0.1	0.1	0.4	0.2
C. diversus (clinical isolate)	0.8	0.4	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.8	0.4
E. coli (ATCC 8739)	0.8	0.4	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.4	0.4
P. aeruginosa (ATCC 9627)	0.8	0.8	0.8	0.4	0.4	0.8	0.8	0.4	0.4	0.8	0.8
P. aeruginosa (clinical isolate)	0.8	0.8	0.8	0.4	0.4	0.8	0.8	0.4	0.4	0.8	0.8
P. fluorescens (food spoilage isolate)	0.8	0.8	0.8	0.4	0.4	0.8	0.8	0.4	0.4	0.8	0.8
S. abony (ATCC 6017)	0.8	0.4	0.4	0.2	0.4	0.4	0.8	0.2	0.2	0.4	0.4
S. abony (clinical isolate)	0.8	0.4	0.4	0.2	0.4	0.4	0.8	0.2	0.2	0.4	0.4

Table 4: Antibacterial activity of several essential oils and main compounds - Minimal Bactericidal Concentration [%].

Test microorganism	CO	CW	JB	MY	α-bisa-bolol	cedrol	(*E,E*)-farnesol	(*E*)-ne-rolidol	(*Z*)-ne-rolidol	sabin-ene	thujop-sene
B. cereus (ATCC 11778)	0.8	0.4	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.4	0.4
S. aureus (ATCC 6538)	0.8	0.4	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.4	0.4
S. aureus (food spoilage isolate)	0.8	0.4	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.4	0.4
S. epidermidis (clinical isolate)	0.8	0.4	0.4	0.2	0.2	0.4	0.8	0.2	0.2	0.4	0.4
C. diversus (clinical isolate)	0.8	0.8	0.4	0.4	0.4	0.8	0.8	0.4	0.4	0.8	0.8
E. coli (ATCC 8739)	0.8	0.8	0.4	0.4	0.4	0.8	0.8	0.4	0.4	0.8	0.8
P. aeruginosa (ATCC 9627)	1	0.8	0.8	0.8	0.8	0.8	>0.8	0.4	0.4	0.8	0.8
P. aeruginosa (clinical isolate)	1	0.8	0.8	0.8	0.8	0.8	>0.8	0.4	0.4	0.8	0.8
P. fluorescens (food spoilage isolate)	1	0.8	0.8	0.8	0.8	0.8	>0.8	0.4	0.4	0.8	0.8
S. abony (ATCC 6017)	0.8	0.8	0.8	0.4	0.8	0.8	>0.8	0.4	0.4	0.8	0.8
S. abony (clinical isolate)	0.8	0.8	0.8	0.4	0.4	0.8	>0.8	0.4	0.4	0.8	0.8

Of all the essential oils tested in this study, to achieve inhibition of microbial growth, cabreuva oil required the highest concentration, which stands in contrast to the results obtained with pure nerolidol, the main constituent of cabreuva oil. (*E*)-and (*Z*)-nerolidol, α-bisabolol and cedrol were shown to reduce bacterial growth significantly and were comparable with myrrh oil, the essential oil with the best antimicrobial activity of those tested.

Experimental

Samples: Essential oil samples for chemical investigation and antimicrobial testing were obtained from Kurt Kitzing GmbH, Wallerstein, Germany. The following references were used: cabreuva oil 801955 lot 15394, cedarwood oil from Texas 801043 lot 17946, essential oil of juniper berries 2x rect. 801116 lot 16785 and essential myrrh oil 800569 lot 16492. To evaluate the antimicrobial activity of the essential oils with respect to their main constituents, *α*-bisabolol, cedrol, (*E,E*)-farnesol, (*E*)-nerolidol, (*Z*)-nerolidol, sabinene and thujopsene (all compounds obtained from Kurt Kitzing, Wallerstein, Germany) were used as pure substances for antimicrobial testing. All samples were subjected to GC analysis, undiluted, with a 0.5 µL plunger-in-needle syringe at a very high split rate.

GC analysis: GC/FID and GC/MS analyses were carried out simultaneously using a Finnigan ThermoQuest TraceGC with a dual split/splitless injector, a FID detector and a Finnigan Automass quadrupole mass spectrometer. One inlet was connected to a 50 m x 0.25 mm x 1.0 µm SE-54 (5% Diphenyl, 1% vinyl-, 95% dimethyl-polysiloxane) fused silica column (CS Chromatographie Service, Germany), the other injector was coupled to a 60 m x 0.25 mm x 0.25 µm Carbowax 20M (polyethylene glycol) column (J & W Scientific, USA). The two columns were connected at the outlet with a quartz Y connector and the combined effluents of the columns were split simultaneously to the FID and MS detectors with a short (ca. 50 cm) 0.1 mm ID fused silica restrictor column as a GC/MS interface. The carrier gas was helium 5.0 with a constant flow rate of 1.5 mL/min., injector temperature was 230°C, FID detector temperature 250°C, GC/MS interface heating 250°C, ion source at 150°C, EI mode at 70 eV, scan range 40 – 300 amu. The following temperature program was used: 46°C for 1 min to 100°C at a rate of 5°C/min.; 100°C to 230°C at 2°C/min; 230°C for 13.2 min. Identification was achieved using Finnigan XCalibur 1.2 software with MS correlations through the NIST 2008 [14], Adams essential oils [15], MassFinder [16] and our own library. Retention indices of reference compounds and from literature data [17 -21] were used to confirm peak data. Quantification was achieved through peak area calculations of the FID chromatogram.

Olfactory evaluation: The samples and reference compounds were olfactory evaluated by two professional perfumers and one aroma chemist. One droplet of each essential oil sample or a reference compound was placed on a commercial odor strip. Furthermore, due to the unavailability of some reference

compounds, evaluations using the GC-split sniffing method were performed. Terms for odor impressions were compared with published data and our private database of reference aroma compounds.

Test microorganisms and preparation of test inoculum: To evaluate the antimicrobial activity, Gram-positive bacteria *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, *S. aureus* (clinical isolate) and *S. epidermidis* (clinical isolate); and Gram-negative bacteria *Citrobacter diversus* (clinical isolate), *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9627, *P. aeruginosa* (clinical isolate), *P. fluorescens* (food spoilage strain, isolated from spoiled food), *Salmonella abony* ATCC 6017, and *S. abony* (clinical isolate) were used as test microorganisms. Test strains were obtained from culture collections of The National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC, Bulgaria), Department "Biochemistry and Microbiology", University of Plovdiv, Bulgaria and Clinic of Infectious Diseases, Medical University of Plovdiv, Bulgaria. Bacteria were maintained on nutrient agar (NA), National Center of Infectious and Parasitic Diseases (NCIPD), Bulgaria.

Overnight bacteria cultures were prepared by inoculating about 2 mL of Mueller-Hinton Broth (MHB, NCIPD, Bulgaria) with 2-3 colonies selected from NA. Broths were incubated at 37°C for 24 h on a rotary shaker at 220 rev/min. Inocula were prepared by diluting overnight cultures by addition of sterile MHB to achieve absorbance corresponding to 0.5 McFarland turbidity standard (1.0/1.5x10^8 CFU/mL) (NCCLS, 1999).

Disc diffusion method: The disc diffusion method was carried out as described by Sacchetti *et al*. [22] in accordance with NCCLS recommendations [23]. The Petri dishes (d = 90 mm), containing solidified MHA (Mueller-Hinton Agar) were inoculated by spreading 100 μL of bacterial inoculum. Sterile paper discs [Whatman 1 (d= 5 mm, NCIPD, Bulgaria)] were soaked with 10 μL of undiluted essential oil and placed on the inoculated surface of the Petri dishes, which were then incubated at 37°C for 24 h. The growth inhibition zone diameter (IZ, mm) was measured to the nearest mm. Results of disc diffusion are solely used for qualitative conclusions.

Serial broth dilution method: The serial broth dilution method was carried out in accordance with NCCLS recommendations [24,25]. A stock solution was prepared by diluting the respective essential oil sample in DMSO (Sigma-Aldrich Co.). Stock solution was then added to culture broth to reach final oil concentrations ranging from 3.28% (v/v) to 0.01% (v/v). Serial dilutions were inoculated with 100 μL of bacteria inoculum, prepared as listed above. The samples were then incubated at 37°C for 24 h and the absorbance was read at 680 nm (CAMSPEC, UK). Control samples of inoculated broth without oil and without DMSO and inoculated broth with DMSO, were also incubated under the same conditions. For the broth dilution method the mean absorbance of the duplicate samples was compared with the mean absorbance of the broth samples containing DMSO without oil to give a measure of the overall reduction in growth. The concentration of DMSO in the broth dilution assay was kept at a concentration to ensure that the effect on bacterial growth was minimal. Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration which resulted in a reduction of > 90% in the observed absorbance. To determine Minimal Bactericidal Concentration (MBC), 100 μL of each dilution showing no growth was spread on MHA. The inoculated Petri dishes were incubated at 37°C for 24 h. The colony forming units were counted and compared with control dishes. MBC was defined as the lowest concentration that killed > 99% of the initial inoculum. Each experiment was performed in duplicate

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