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SHORT COMMUNICATION

Antimicrobial Activity of Clove and Rosemary Essential Oils Alone and in Combination

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In the present study, the antimicrobial activity of the essential oils from clove (*Syzygium aromaticum* (L.) Merr. et Perry) and rosemary (*Rosmarinus officinalis* L.) was tested alone and in combination. The compositions of the oils were analysed by GC/MS. Minimum inhibitory concentrations (MIC) against three Gram-positive bacteria, three Gram-negative bacteria and two fungi were determined for the essential oils and their mixtures. Furthermore, time-kill dynamic processes of clove and rosemary essential oils against *Staphylococcus epidermidis, Escherichia coli* and *Candida albicans* were tested. Both essential oils possessed significant antimicrobial effects against all microorganisms tested. The MICs of clove oil ranged from 0.062% to 0.500% (v/v), while the MICs of rosemary oil ranged from 0.125% to 1.000% (v/v). The antimicrobial activity of combinations of the two essential oils indicated their additive, synergistic or antagonistic effects against individual microorganism tests. The time-kill curves of clove and rosemary essential oils towards three strains showed clearly bactericidal and fungicidal processes of $^1/_2 \times$ MIC, MIC, MBC and 2 × MIC.

Keywords: antimicrobial; in combination; essential oil; clove; rosemary.

INTRODUCTION

Commercial antimicrobial drugs have been commonly employed as treatment for infectious diseases for many years, however, in recent years, the indiscriminate use of these antibiotics has developed multiple resistances and side effects. Therefore, more natural antimicrobial substances from plants are desired. A large number of herbs possess antimicrobial activity (Voravuthikunchai *et al.*, 2004; Mothana and Lindequist, 2005), and some active components of them have become a potential source of new antiinfective agents (Agunu *et al.*, 2005; Buwa and van Staden, 2006).

Essential oils, herbal extracts, are well known for their antimicrobial activity (Friedman *et al.*, 2002; Kalemba and Kunicka, 2003). They are widely used in medicine and the food industry for this purpose. The essential oils from clove (*Syzygium aromaticum* (L.) Merr. et Perry) and rosemary (*Rosmarinus officinalis* L.) are both natural substances that are not harmful when consumed in medicine and food products. There have been some reports on the essential oils activity of clove and rosemary that inhibited the growth of bacteria and fungi. The antimicrobial properties of clove essential oil was tested and showed inhibitory activity to *Listeria monocytogenes, Campylobacter jejuni, Salmonella enteritidis, Bacillus cereus, Escherichia coli* and *Staphylococcus aureus* (Cressy *et al.*, 2003; Velluti *et al.*, 2003). Rosemary essential oil is also used as an antibacterial and antifungal agent (Valero and Salmerón, 2003).

In the present study, the antimicrobial activity of clove and rosemary essential oils was investigated against a range of microorganisms alone and in combination. The inhibition diameter, MICs, MBCs and the time-kill dynamic procedures of clove and rosemary essential oils were tested, respectively. The analysis of bactericidal and fungicidal activity provides detailed information on changes in colony counts over time. Furthermore, the antimicrobial activity of mixtures of the two essential oils were tested, which has not been reported before. Combinations of individual oils may lead to additive, synergistic or antagonistic effects (Delaquis *et al.*, 2002), which raise industrial interest in naturally produced medical products and food preservatives. This report provides a basis for further exploitation and use of the two plant resources on human health and food safety.

MATERIALS AND METHODS

Essential oils and positive controls. Clove essential oil was purchased from NanYang Zhang Zhongjing Digital Chinese Medicine Co., Ltd in the HeNan Province of China. Fresh, air-dried leaves of rosemary were collected from the Fu Yang base of ZheJiang

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HiSun Pharmaceutical Co., Ltd in China. Authenticated voucher specimens were deposited at the herbarium, Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin, China. Rosemary essential oil was isolated by steam distillation from the rosemary powdered leaves for 3 h and then was dried over anhydrous sodium sulphate. The essential oil was filtered for further processing.

Streptomycin and amphotericin B (Sigma) were used as a bacterial positive control and a fungi positive control, respectively.

Microorganisms and cultural methods. *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Proteus vulgaris* (ATCC 49132), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 8739), *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231) were tested. These strains were obtained from the Institute of Applied Microbiology, HeiLongJiang Academy of Science, China. They were maintained on an agar slant at 4 °C. All strains were activated in nutrient agar at 37 °C for 24 h, except for *Aspergillus niger* which was incubated in PDA at 25 °C for 5 days before testing.

Chemical analysis. Clove and rosemary essential oils were analysed by GC/MS. The analyses were performed with a VG Platform II spectrometer hyphenated instrument (UK). The gas chromatography was equipped with a fused silica capillary column (30 m × 0.25 mm i.d.; film thickness, 0.25 μm). The carrier gas was helium at 1.0 mL/min. The temperature of the column was programmed from 50 to 240 °C at 8 °C/min. The injection port and detector were set at 250 °C. The oil components were identified by comparison of their retention indices and mass spectra with a NIST Mass Spectral Library. The percentage content was calculated by peak area normalization.

Sensitivity test of the essential oils. The agar disc diffusion method was employed to determine the sensitivity of the essential oils (NCCLS, 2002). The solid media plates were swabbed with the respective suspension (10^8 CFU/mL) and kept for 2 h at 4 °C for absorption. Filter paper discs (6.0 mm in diameter) were impregnated with 5 μL essential oils and placed on the agar surface. Streptomycin (1.2 mg/mL) 20 μL was used as a positive bacterial control, Amphotericin B (1.0 mg/mL) 20 μL was used as a positive control of fungi and sterilized physiological saline solution (0.9% w/v) 20 μL was used for a negative control. All plates were incubated under proper conditions as described above. All experiments were performed in triplicate.

MIC and MBC determination of clove and rosemary essential oils. The estimation of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were measured by the broth microdilution method (NCCLS, 2002). The essential oils were individually dissolved in sterilized physiological saline solution (0.9% w/v) supplemented with Tween 80 (Sigma) at a final concentration of 0.5% (v/v). Serial doubling dilutions of the oils were prepared in a 96-well microtiter plate in the range 0.156% to 4.000% (v/v). Each essential oil dilution (100 μL) was dispensed into the wells of a microtiter plate, each well was then inoculated with 100 μL of the suspension. The resulting suspensions were mixed with a micro-pipettor. The final concentration of each strain was adjusted to 10^5–10^6 CFU/mL. All microtiter plates against all microorganisms were incubated at 37 °C for 24 h, except for *Aspergillus niger* which was incubated at 25 °C for 5 days. After incubation, the wells were examined for growth of microorganisms and the MICs were determined. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The MBCs were confirmed by reinoculating on agar plates with 10 μL of each culture medium from the microplates. The MBC is defined as the lowest concentration of the essential oil at which incubated microorganisms are completely killed. Streptomycin and amphotericin B served as positive controls in parallel experiments. Each experiment was repeated three times.

MICs determination of mixture of essential oils. Mixtures of clove and rosemary essential oils at ratios of 1:1, 1:3, 1:5, 1:7, 1:9, and 3:1, 5:1, 7:1, 9:1 were tested for MICs with the broth microdilution method.

Time-kill studies. Time-kill dynamic procedures were performed as described by Avila *et al.* (1999). *Staphylococcus epidermidis*, *Escherichia coli* and *Candida albicans* were tested to investigate the time-kill plots of clove and rosemary essential oils, respectively. The final concentration of suspension of the strains was adjusted to 10^5–10^6 CFU/mL. Essential oil concentrations used in the test solutions were equivalent to 0.5, 1 and 2 × MIC and MBC, respectively. After incubating for 0, 1, 2, 4, 8, 12, 24 and 30 h with the broth micro dilution method, 50 μL liquid was removed from the test solution for tenfold serial dilution. Thereafter, 25 μL liquid from each dilution was spread on the surface of nutrient agar plates and incubated at 37 °C for 24 h and *Candida albicans* incubated for 48 h, then the number of CFU/mL was counted. Each assay included a growth control with no essential oil. Experiments were carried out in triplicate. Time-kill curves were constructed by plotting $\log_{10}$ CFU/mL against time (h).

RESULTS

Chemical composition of the essential oils

The results of chemical analysis of the essential oils were as follows. The relative contents of the main compositions in clove essential oil were eugenol (68.52%), *β*-caryophyllene (19.00%), 2-methoxy-4-[2-propenyl] phenol acetate (10.15%) and *α*-caryophyllene (1.85%). In rosemary essential oil, the main compositions were 1, 8-cineole (27.23%), *α*-pinene (19.43%), camphor (14.26%), camphene (11.52%), borneol (3.17%), *β*-caryophyllene (2.41%) and bornyl acetate (1.13%).

Sensitivity of the essential oils

The sensitivity of the clove and rosemary essential oils against bacteria and fungi studied by the disc diffusion method is shown in Table 1. Both essential oils

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Table 1. Inhibition diameters of clove and rosemary essential oils against bacteria and fungi

Strain		Inhibition diameter (mm)		
		Clove essential oil	Rosemary essential oil	Positive control
Bacteria	*Staphylococcus epidermidis*	16.8 ± 1.2	10.8 ± 0.3	17.5 ± 0.5
	Staphylococcus aureus	16.3 ± 0.7	18.5 ± 1.3	20.0 ± 0.8
	Bacillus subtilis	19.5 ± 0.5	13.0 ± 1.0	25.0 ± 0.7
	Escherichia coli	16.3 ± 1.3	10.0 ± 0.8	16.0 ± 1.5
	Proteus vulgaris	18.2 ± 1.3	12.3 ± 1.0	17.5 ± 1.0
	Pseudomonas aeruginosa	9.5 ± 0.5	6.0 ± 0	6.0 ± 0
Fungi	*Candida albicans*	32.0 ± 1.0	21.5 ± 1.5	22.5 ± 1.0
	Aspergillus niger	40.0 ± 0.5	14.0 ± 0.7	18.0 ± 1.5

Streptomycin as a positive control of bacteria; amphotericin B as positive control of fungi.

exhibited significant susceptibility, with >10 mm inhibition diameter towards *Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Proteus vulgaris*. The effect on *Pseudomonas aeruginosa* was weak (<10 mm inhibition diameter). For fungi, clove essential oil exhibited higher sensitivity than rosemary essential oil. Clove essential oil exhibited the best antifungal activity against *Candida albicans* and *Aspergillus niger.* The inhibition diameters were 32.0 mm and 40.0 mm, respectively. The antibacterial activity of the essential oils was similar to that of streptomycin. For fungi, the inhibition diameters of clove essential oil were higher than that of amphotericin B, while the inhibition diameters of rosemary essential oil were similar to that of amphotericin B.

MICs and MBCs of clove and rosemary essential oils

As can be seen in Tables 2 and 3, both essential oils exhibited inhibitory effects towards all the test organisms. Clove essential oil exhibited a little higher antimicrobial activity than that of rosemary essential oil, which was similar to the results of the sensitivity test (Table 1). The antimicrobial activity of rosemary essential oil against *Pseudomonas aeruginosa* and *Aspergillus niger* was less than against the other bacteria and *Candida albicans.* The MICs for the clove essential oil ranged from 0.062% (v/v) to 0.500% (v/v) for all test microorganisms, while MICs for rosemary oil ranged from 0.125% (v/v) to 1.000 % (v/v), MBC values of the two oils were similar or even higher than the corresponding MIC values.

MICs of the mixture of the essential oils

This study focused on the antimicrobial activity of mixtures of clove and rosemary essential oils. The MIC results (Table 4) showed that as the concentration of clove essential oil was increased in the overall oil composition, the antimicrobial activity increased

Table 2. MICs and MBCs of clove and rosemary essential oils against bacteria

Bacteria	Positive control streptomycin (μg/mL)		Clove essential oil (%v/v)		Rosemary essential oil (%v/v)	
	MIC	MBC	MIC	MBC	MIC	MBC
Se	7.5	120.0	0.250	0.250	0.250	0.500
Sa	30.0	>120.0	0.125	0.250	0.125	0.250
Bs	30.0	120.0	0.125	0.250	0.125	0.500
Ec	60.0	>120.0	0.125	0.125	0.250	0.500
Pv	15.0	120.0	0.125	0.250	0.250	0.500
Pa	120.0	>120.0	0.500	0.500	1.000	2.000

Se, *Staphylococcus epidermidis*; Sa, *Staphylococcus aureus*; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Pv, *Proteus vulgaris*; Pa, *Pseudomonas aeruginosa.*

Table 3. MICs and MBCs of clove and rosemary essential oils against fungi

Fungi	Positive control amphotericin B (μg/mL)		Clove essential oil (%v/v)		Rosemary essential oil (%v/v)	
	MIC	MBC	MIC	MBC	MIC	MBC
Ca	0.195	0.195	0.125	0.250	0.250	0.500
An	0.780	0.780	0.062	0.125	1.000	>2.000

Ca, *Candida albicans*; An, *Aspergillus niger.*

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Table 4. MIC values (as %, v/v) of clove essential oil and rosemary essential oil blends

	Organism							
A:B	Se	Sa	Bs	Ec	Pv	Pa	Ca	An
1:0	0.250	0.125	0.125	0.125	0.125	0.500	0.125	0.062
0:1	0.250	0.125	0.125	0.250	0.250	1.000	0.250	1.000
1:1	0.250	0.125	0.125	0.125	0.125	1.000	0.125	0.125
1:3	0.250	0.125	0.125	0.250	0.250	1.000	0.125	0.250
1:5	0.250	0.125	0.125	0.250	0.250	1.000	0.125	0.500
1:7	0.250	0.125	0.125	0.250	0.250	1.000	0.125	1.000
1:9	0.250	0.125	0.125	0.250	0.250	1.000	0.125	1.000
3:1	0.125	0.125	0.125	0.125	0.125	0.500	0.031	0.062
5:1	0.125	0.125	0.125	0.125	0.125	0.500	0.031	0.062
7:1	0.125	0.125	0.125	0.125	0.125	0.500	0.062	0.062
9:1	0.125	0.125	0.125	0.125	0.125	0.500	0.062	0.062

A, clove essential oil; B: rosemary essential oil.
Se, *Staphylococcus epidermidis*; Sa, *Staphylococcus aureus*; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Pv, *Proteus vulgaris*; Pa, *Pseudomonas aeruginosa*; Ca, *Candida albicans*; An, *Aspergillus niger.*

accordingly. Combinations of the two essential oils exerted additive antimicrobial effects against *Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa.* A synergistic effect was observed for *Candida albicans*, whereas an antagonism was found for *Aspergillus niger* when the ratios of clove essential oil and rosemary essential oil were 1:5, 1:7 and 1:9.

Time-kill curves

The time-kill dynamic process of the essential oils towards three microorganisms are presented in Fig. 1 and the results show minor differences. At $^1/_2 \times$ MIC, bacterial growth was inhibited with no noticeable drop in bacterial counts in the first 4–8 h, then, the colony counts continued to grow until about 10^7–10^8 CFU/mL. At 1 × MIC (when MIC is not identical to MBC), bacterial growth was finally inhibited at a lower level and the colony counts remained near the initial starting concentration after 30 h. At MBC, both essential oils had a lethal effect on *Staphylococcus epidermidis* and *Escherichia coli* within the first 12 h, while there was a complete eradication against *Candida albicans* before 8 h. However, at 2 × MIC (when 2 × MIC was not identical to MBC), both essential oils showed a complete bacterial eradication effect within the first 2 h (Fig. 1 A, C, F). The colony numbers of the control were still increasing within 30 h.

DISCUSSION AND CONCLUSION

The MICs and MBCs of clove and rosemary essential oils in this study were similar to the known literature (Hili *et al.*, 1997; Del Campo and Amiot, 2000), with a little difference, which could be for several reasons such as a different growing environment of clove and rosemary, different extraction methods of essential oils, and so on. Different essential oils have different antimicrobial activity because of their components (Ricci *et al.*, 2005). The antimicrobial activity of clove essential oils could be associated with eugenol, the main component of clove oil, which is already known to exhibit antibacterial and antifungal activity (Suresh *et al.*, 1992). The main compounds present in rosemary essential oil were 1,8-cineole, α-pinene, camphor, etc., which have been evaluated for their antimicrobial effects (Viljoen *et al.*, 2003).

The antimicrobial activity of combinations of clove and rosemary essential oils has not been reported before. Individual essential oils contain complex components which, when combined with each other, may lead to additive, synergistic or antagonistic effects. The results showed that combinations of clove and rosemary essential oils exhibited additive or synergistic effects against all the test strains, with the exception of *Aspergillus niger*. The mechanism of antimicrobial activity of mixed essential oils is still not clear and further studies in this area are needed. This result may be useful for the combination of clove and rosemary essential oils against special microorganisms in medicine and the food industry.

Time-kill curves of clove and rosemary essential oils against three microorganisms have not been reported before. It was found that the low concentrations of both essential oils were not sufficient to kill significant microorganisms. At MBC and 2 × MIC, they had a lethal effect on the microorganisms and had prolonged antimicrobial activity. Because the speed of growth of microorganisms and components of the essential oils are different, time-kill curves against each strain presented different figures.

The present study showed that both essential oils possessed a wide spectrum of antimicrobial activity against all the microorganisms tested. A combination of clove and rosemary oil exerted additive, synergistic or antagonistic effects depending on the corresponding microorganism. The underlying modes of action remain to be explored in the future. The fact that both essential oils alone or in combination exhibited antimicrobial activities against the microorganisms studied supports the utilization of these plants in the treatment of pathogenesic diseases as well as their use as food preservatives.

Based on these data, further chemical and pharmacological investigations are required for clove and

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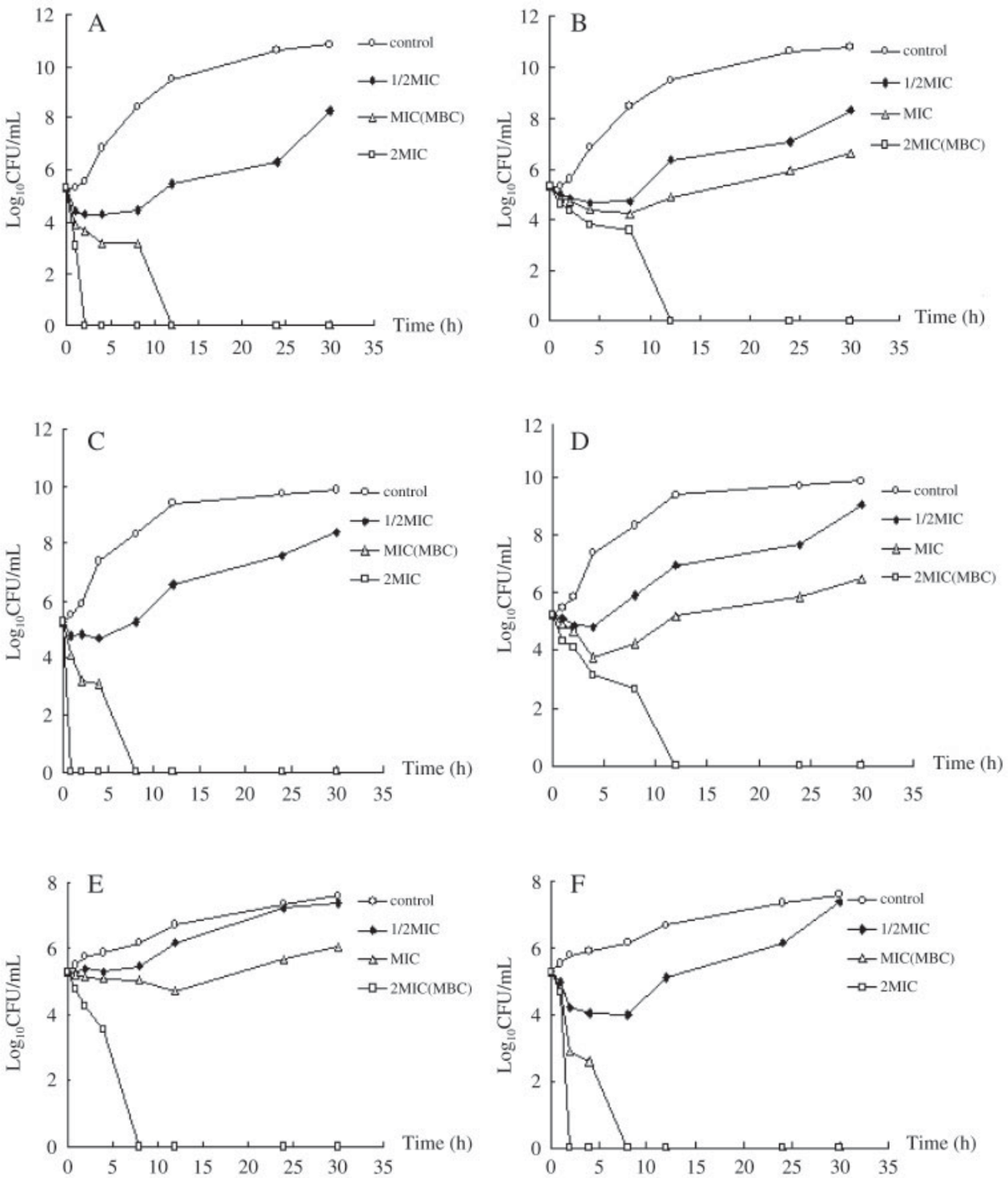

Figure 1. Time-kill plots of clove oil (A, C, E) and rosemary oil (B, D, F) against three species of *Staphylococcus epidermidis* (A and B), *Escherichia coli* (C and D), *Candida albicans* (E and F). The concentrations used for the test: $^1/_2$×MIC, MIC, 2×MIC and MBC, the control tube did not contain essential oil.

rosemary essential oils. The *in vitro* results of the present investigation provide evidence that the two essential oils represent a potentially rich source for medicine and food preservatives against microorganisms. Hence, essential oils used alone or in combination may be useful as alternative antiinfectious agents and as food preservatives.

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