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Combination of essential oils for the application in antimicrobial food packaging

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Abstract: Antimicrobial food packaging not only meets traditional packaging requirements, but also is able to kill pathogenic or spoilage microorganisms which are contaminating foods caused by defective packaging. Natural spice essential oils are well-known antimicrobial agents that could be used as food preservation to inhibit microbial growth. The manuscript provides evidence that some of these essential oils may be effective as indirect food additives combined into food packaging materials. In present study, agar diffusion methods was used to evaluate the antimicrobial activities of 10 spice essential oils against several common bacterial and fungal contaminants of foods, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. The antimicrobial test results showed that the essential oils of oregano and basil had strong antimicrobial activity and significant broad spectrum against the test microorganisms. It was also worth noting that the essential oil of perilla strongly inhibited the growth of *Saccharomyces cerevisiae*, and the essential oil of bergamot was active against *Staphylococcus aureus* and *Bacillus subtilis*. Moreover, chemical compositions of these effective essential oils were analyzed by GC/MS. The effect of various combinations of these essential oils on antimicrobial activity has also been examined, and the combinations had higher inhibitory effect towards test microorganisms than that of individual essential oil. The application of combined essential oils-based active packaging in preservation of strawberry was also investigated. The use of active packaging possessed significant fresh-keeping effects on sensory, microbial value compared to the control testing, and the product shelf-life increased. These results suggest that combined essential oils-based antimicrobial food packaging is a promising food technology to ensure the quality and safety of food products.

Keywords: Essential oils; Food-borne pathogens; GC/MS; Synergy; Antimicrobial food packaging; Strawberry

1. Introduction

In spite of modern improvements in food production and preservation techniques, such as genetic engineering, irradiation of food, and modified-atmosphere packaging (WHO, 2002), food safety is a growing public health problem. The survival of microorganisms in foods is an important problem that may lead to spoilage, formation of toxins and quality deterioration of food products (Celiktas et al., 2007). Preservative can be used of food preservation, however, some modern synthetic preservatives have become controversial because they have been shown to cause respiratory or other health problems. Therefore, it is necessary to find a novel way to reduce or eliminate food-borne pathogens during the shelf life of food products. In the meantime, the increasing demand of consumers for natural products, has led to research on new antimicrobial agents from plants to improve the safety of products (Goni et al., 2009).

Essential oils (EOs) are the volatile oily liquids of the secondary metabolism of scented plants, which are obtained from different plant parts, such as flowers, leaves, seeds, bark, fruits and roots (Burt, 2004). Though some essential oils have been long researched for their antibacterial, antifungal, antiviral, and antioxidant properties (Mourey, & Canillac, 2002; Kordali *et al.*, 2005; Sylvestre et al., 2006), the recent enhancement of interest in 'green' consumerism has given rise to a renewal scientific awareness of them. Due to the excellent antimicrobial function of essential oils, they possess great potential as natural additives for food preservation.

Antimicrobial packaging is a form of active packaging that could extend the shelf-life of product and provides microbial safety for consumers. Several compounds have been proposed for antimicrobial activity in food packaging, including natural antimicrobial agent such as spices EOs (Tharanathan, 2003). In order to maintain high concentrations of preservatives on the food surfaces, preservative solution which added to EOs can be applied. Even though desired antimicrobial activity of several essential oils against pathogenic and spoilage microorganisms are performed in vitro test, it has generally been found that a higher concentration is needed to achieve the same effect in foods (Shelef et al., 1984). This fact may lead to an organoleptic impact as the use of natural aromatic preservatives can alter the taste of food and exceeds the flavor threshold acceptable to consumers (Hsieh *et al.*, 2001; Nazer *et al.*, 2005). To avoid this problem, the synergistic effects of complex essential oils may help to obtain effective antimicrobial activity at sufficiently low concentrations and consequently reduce sensory impact (Gutierrez et al., 2009).

The aims of the present study were: (1) to screen the antimicrobial properties of ten spice essential oils against four common pathogens and food spoilage bacteria and

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yeasts, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*; (2) to access synergistic, additive or antagonistic effects of selected complex essential oils at low doses by checkerboard tests and (3) to determine the fresh-keeping efficacy of preservative solution incorporated with these essential oils on strawberry.

2. Material and methods

2.1. Essential oils and fruit material

The essential oils, Patchouli oil (Pogostemon cablin), Clary Sage oil (Salvia sclarea L.), Rosemary oil (Rosmarinus officinalis), Basil oil (Ocimum basilicum), Spearmint oil (Mentha spicata), Oregano oil (Origanum vulgare), Perilla oil (Perilla arguta), Absinthe oil (Artemisia absinthiuml), Bergamot oil (Citrus bergamia) and Lavender oil (Lavandula angustifolia), were supplied by Guanxiang Chemicals Trading co ltd. (Changsha, China). The fresh crushed leaves and flowers of these plants were subjected to steam distillation for 3 h. The essentials oils obtained were dried over anhydrous sodium sulfate and stored at low temperature (4-6 °C) before use for analysis.

Strawberry fruits were obtained from a local market and selected for uniformity in size, appearance, ripeness and the absence of physical defects. The selected fruits were randomised before being used for essential oil treatments.

2.2. Antimicrobial activity

2.2.1 Microbial strains and growth conditions

A panel of food-borne pathogenic microorganisms were used to assess the antimicrobial properties, including the Gram-positive *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, Gram-negative *Escherichia coli* ATCC 8099, and the yeast *Saccharomyces cerevisiae* ATCC 9763. All strains were obtained from China General Microbiological Culture Collection Center and were maintained on slants of Nutrient Agar for bacteria and YPD Agar for the yeast at 4 °C.

Active cultures for experimental use were prepared by transferring a loop of cells from the agar slant to a test tube containing 5 ml of Nutrient Broth for bacteria and YPD Broth for the yeast, respectively. They were then incubated overnight at 37 °C for bacteria and 30 °C for the yeast. The turbidity of the cell suspensions was measured at 600 nm and adjusted to the required concentration of 10^5-10^6 CFU/ml using the McFarland standard.

2.2.2 Agar disc diffusion assay

The essential oils were screened for antimicrobial activity using the agar disc diffusion method (Rota et al., 2004) against 4 microorganisms. Nutrient Agar and YPD Agar were sterilised in an autoclave and cooled to 45-50 °C before being poured into 90 mm Petri dishes. After solidifying, sterile blank filter discs (6 mm diameter) containing 5µl of each the essential oil were applied to the surface of agar plates that were previously seeded by spreading of 200 µl overnight fresh inoculums suspension. One standard antibiotic and three chemical preservatives were used as positive control: kanamycin sulfate (30 µg/disc), sodium benzoate (200 µg/disc), potassium sorbate (200 µg/disc) and methylparaben (200 µg/disc), while sterile water was used as a negative control. The inoculated plates were incubated for 24 h at 37 °C for bacterial and 48 h at 30 °C for yeast, respectively. Microbial inhibition was visually appraised as the diameter of the inhibition zones surrounding the discs (disk diameter included) and recorded in millimeter. The diameters of the inhibition zones were measured with a digital caliper. Three agar disc diffusion tests were carried out on each microorganism and each test was performed in duplicate.

2.2.3 Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) is cited by the most researchers as a measure of the antimicrobial performance of EOs. Bacteria and yeast sensitive to the essential oils in disc diffusion assay were studied for their MIC values with some modifications of the method described by Weerakkody et al. (2010). The inoculums were prepared from overnight broth cultures and suspensions were adjusted to the required microbial density (1×10^8 CFU/ml) using an Ultraspec 2000 spectrophotometer at 600 nm. After adding 20 µl of EOs to the first tube containing 4 ml of broth, serial two-fold dilutions were made in a concentration range of 0.039-5 µl/ml in 10 ml sterile test tubes containing Nutrient broth for bacteria and YPD broth for the yeast. A 400 µl suspension (1×10^8 CFU/ml) of tested microorganisms was added to each tube. A negative control tube contained broth and microorganism and a positive control tube contained 50 µg/ml of kanamycin sulfate in broth and microorganism was maintained. The minimum inhibitory concentration (MIC) was defined as the concentration in the lowest serial dilution of the essential oils which resulted in the lack of visible microorganism growth in tubes after 24 h (bacteria) and 48 h (yeast).

2.3 Gas chromatography/mass spectrograph (GC/MS) analysis of selected essential oils

The selected essential oils were analyzed by Agilent 7890A/5975C GC-MS system, equipped with a DB-624 capillary column (30 m×0.25 mm; film thickness, 0.25 µm). The oven temperature program was initiated at 60 °C, held for 3 min then raised up to 220 °C at a rate of 5 °C /min held for 1 min. The injector temperature was 250 °C. The amount of injection was 0.2 µl. Helium was used

as carrier gas at flow rate of 1 ml/min, with a split ratio equal to 1:4. The MS was operated in EI ionization mode at 70 ev, and complete scans from 40 to 350 amu (atomic mass units) were recorded. Compounds were identified by the comparison of their mass spectra with those of NIST commercial library. All analyses were carried in triplicate.

2.4 Synergetic effect testing: checkerboard method

The broth dilution checkerboard method, which is frequently used to assess interactive inhibition in vitro, was used to determine the antimicrobial effect of selected complex essential oils obtained in antimicrobial activity testing. The assay was arranged as follows: EO_A was diluted two-fold in vertical orientation, while EO_B was diluted two-fold in horizontal orientation. The concentrations of EO_A and EO_B prepared corresponded to 1/2, 1/4 and 1/8 of the MIC values, respectively. Subsequently, 400 µl suspension containing 1×10^8 CFU/ml of the indicator strain was added to each tube. The inoculated tubes were incubated overnight at 37 °C for bacteria and 30 °C for the yeast, and then evaluated for microbial growth.

The checkerboard method is often combined with calculation of fractional inhibitory concentration (FIC) indices to assess effects of combinations. The FIC indices (FICI) were calculated as $FIC_A + FIC_B$ (Hemaiswarya et al., 2008), where FIC_A = (MIC_A combination / MIC_A alone) and FIC_B = (MIC_B combination / MIC_B alone). The results were interpreted as synergy (FICI < 0.5), addition (0.5 ≤ FICI ≤ 1), indifference (1 < FICI ≤ 4) or antagonism (FICI > 4).

2.5 Fresh-keeping of strawberry by preservative solution

Microbial growth can seriously limit the safety and shelf life of fruits and there is a potential risk for a wide range of raw fruits to become contaminated with microorganisms, including human pathogens. In our study, strawberries were chosen for preserve test due to they were prone to microbial spoilage. For preparing preservative solution, the concentration of the complex essential oils were prepared at MIC value by dissolving its requisite amount in 5 ml 5% tween-80 and then admixing with 95 ml distilled water. Fresh strawberries were washed before treatments. Fruits were dipped into the preservative solution twice for 30 s each time and then under a fan about 20min to ensure dryness following the dipping. The control group was kept parallel to the treatment without EOs. Strawberries were put on a tray and kept in the shade. The checkpoints were scheduled for 0 and 3 days and then the degree of infection on fruits was visually evaluated.

3 Results and discussion

3.1 Antimicrobial activity

Compared with those of kanamycin sulfate, sodium benzoate, potassium sorbate and methylparaben used as positive controls, initially screening of the antimicrobial activity of the investigated essential oils was studied against four tested microorganisms using the agar disc diffusion assay, which was assessed by the presence and absence of inhibition zones. The antimicrobial activity of those essential oils can be classified into weak activity (inhibition zone ≤ 12 mm), moderate activity (12 mm < inhibition zone < 20 mm) and strong activity (inhibition zone ≥ 20 mm). The diameter of the inhibition zones of tested essential oils are shown in Table 1.

The essential oils displayed a variable degree of antimicrobial activity against the different strains tested. The oregano oil showed high activity and significant broad spectrum against all the tested microorganisms, especially with the zones of inhibition for both Gram-positive bacteria and yeast greater than 27 mm. It was also worth noting that the essential oils of basil and bergamot were active against the Gram-positive bacteria (*S. aureus* and *B. subtilis*) as they were more effective than 30 µg of kanamycin sulfate, meanwhile the essential oil of perilla strongly inhibited the growth of the yeast, *S. cerevisiae*. Generally, the Gram-negative bacterium *E. coli* was found to be the most resistant to the examined essential oils, only moderate inhibition (15-19 mm) was observed for oregano oil and basil oil, and this trait had been attributed to the external lipopolysaccharide wall that surrounds the peptidoglycan cell wall of the former. Comparatively, essential oils from patchouli, clary sage, rosemary, spearmint, absinthe and lavender showed weak or moderate activity for tested stains, with the inhibition zones less than 16 mm.

Following these tests, oregano, basil, bergamot and perilla essential oils were selected for further study on MICs as described previously. The MICs of the selected EOs against the tested strains are presented in Table 2. As shown in the table, the oils have variable levels of inhibition. The MICs values confirmed the results obtained with the agar disc diffusion method. Oregano oil had the lowest MICs (0.625 µl/ml) for all the tested microorganisms, while perilla oil exhibited the same strong effectiveness (MIC = 0.625 µl/ml) against the yeast, *S. cerevisiae*. Basil oil and bergamot oil displayed high effect against the Gram-positive bacteria, with MICs of 1.25µl/ml against *S. aureus*, while the MICs of 0.625 µl/ml and 1.25 µl/ml against *B. subtilis*, respectively.

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3.2 Gas chromatography/mass spectrograph (GC/MS) analysis of selected essential oils

The chemical composition of oregano, basil, bergamot and perilla oils was then analyzed using a GC-MS technique. As shown in Fig.1, the differences in their composition are clear. Table 3 shows the main chemical components of tested essential oils, together with the retention time of the compounds. It is noteworthy that the phenols and terpenes were the main group of constituents in oregano and basil oils. The major constituents of oregano oil were carvacrol (29.85%), p-cymene (15.10%), γ-Terpinen (12.52%) and thymol (8.79%), while the basil oil contained eugenol (62.97%) followed by caryophyllene (21.57%). In the case of bergamot oil, bergamol (16.00%), linalool (13.97%) and D-limonene (13.35%) were found as the main volatile constituents. In comparison, the dominant components of perilla oil were mainly ketones, such as 2-Pentanoylfuran, reaching percentages of 23.03%.

The antimicrobial activity of essential oils can be attributed to the presence a number of small phenols, terpenes and aldoketones (Panizzi et al., 1993; Ceylan, & Fung, 2004), which also have been shown to exhibit antimicrobial activity in pure form. Therefore, a higher antimicrobial activity of oregano, basil, bergamot and perilla essential oils could be explained by a significant amount of carvacrol (29.85%), eugenol (62.97%), D-limonene (13.35%) and 2-Pentanoylfuran (23.03%), respectively. A number of compounds present in relatively low concentrations, such as α-pinene, β-pinene, thymol, γ-terpinen, terpinolene, piperitone and perillene, could also be expected to make a significant contribution to the antimicrobial activity of the tested essential oils. On the other hand, it is worth noting that the composition content of test essential oils in this study differed greatly from other previous reports. This difference may due to analytical techniques, chemotypes, culture climate and other culture conditions, which may affect biological activities (Calvey et al., 1998).

3.3 Synergetic effect testing: checkerboard method

To explore the possibility of developing a more effective antimicrobial activity, we extended our investigation to study the synergistic effect among the selected essential oils. On the basis of previous research (Davidson, & Parish, 1989), synergism is observed when the effect of the combined substances is greater than the sum of the individual effects; additive effect is observed when the combined effect is equal to the sum of the individual effects; antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. According to the checkerboard test, the FIC indices for the complex EOs are shown in Table 4. With reference to the FICI scale, all of the tested combinations displayed a synergistic activity (FICI = 0.375) for *S. aureus* in this study. Combinations of oregano with basil or bergamot were more effective against *B. subtilis*, which exhibited a useful addictive effect with a FICI of 0.75. The combinations of oregano with basil and oregano with parilla also had additive effects (FICI = 0.75) against *E. coli* and *S. cerevisiae*, respectively. No indifference and antagonism was observed for any of the combinations evaluated. Based on an overall consideration of antimicrobial activity, organoleptic impact and cost, the kinds and MIC values of complex essential oils we selected were listed as follows: oregano-basil (0.313-0.313 μl/ml) for *E. coli*, basil-bergamot (0.625-0.156 μl/ml) for *S. aureus*, oregano-bergamot (0.313-0.156 μl/ml) for *B. subtilis* and oregano-perilla (0.313-0.156 μl/ml) for *S. cerevisiae*.

The antimicrobial activity of essential oils would be related to the respective composition and structural configuration of the plant volatile oils, their functional groups and possible synergistic interactions between components (Dorman, & Deans, 2000). Some studies have concluded that whole EOs have a greater antibacterial activity than the major components mixed (Gill et al., 2002). Similarly, Burt (2004) and Ultee et al. (2000) also suggested that the minor components present in the EOs are more critical to the activity than EOs main components mixed, and the combination of major components with other minor components that have a weaker activity may achieve a synergistic effect. Previous studies reported that the acetate compounds may positively influence the activity of marjoram EO (Dorman, & Deans, 2000). The general components of many plants EOs, which are camphor and eucalyptol, possess oxygen functions in their structure and these functions are indentified to increase the antimicrobial activities of terpenoids (Naigre et al., 1996). As the interaction of EO compositions mentioned above, the mixture of EOs which consists of different biochemical components may increase the antimicrobial efficacy distinctly.

3.4 Fresh-keeping of strawberry by preservative solution

In order to develop the new technique of strawberry storage quality at room temperature, the fresh-keeping effects of preservative solution contained complex EOs on strawberry was investigated, which compared with untreated control. As shown in Fig.2, fungal colonies are clearly present on the strawberries to the controls after 5 days storage, but this signs is not visible in the strawberries dipped with the preservative solution. Therefore, active preservative solution for fresh-keeping of strawberries was feasible, senescence of fruit delayed and the storage time prolonged.

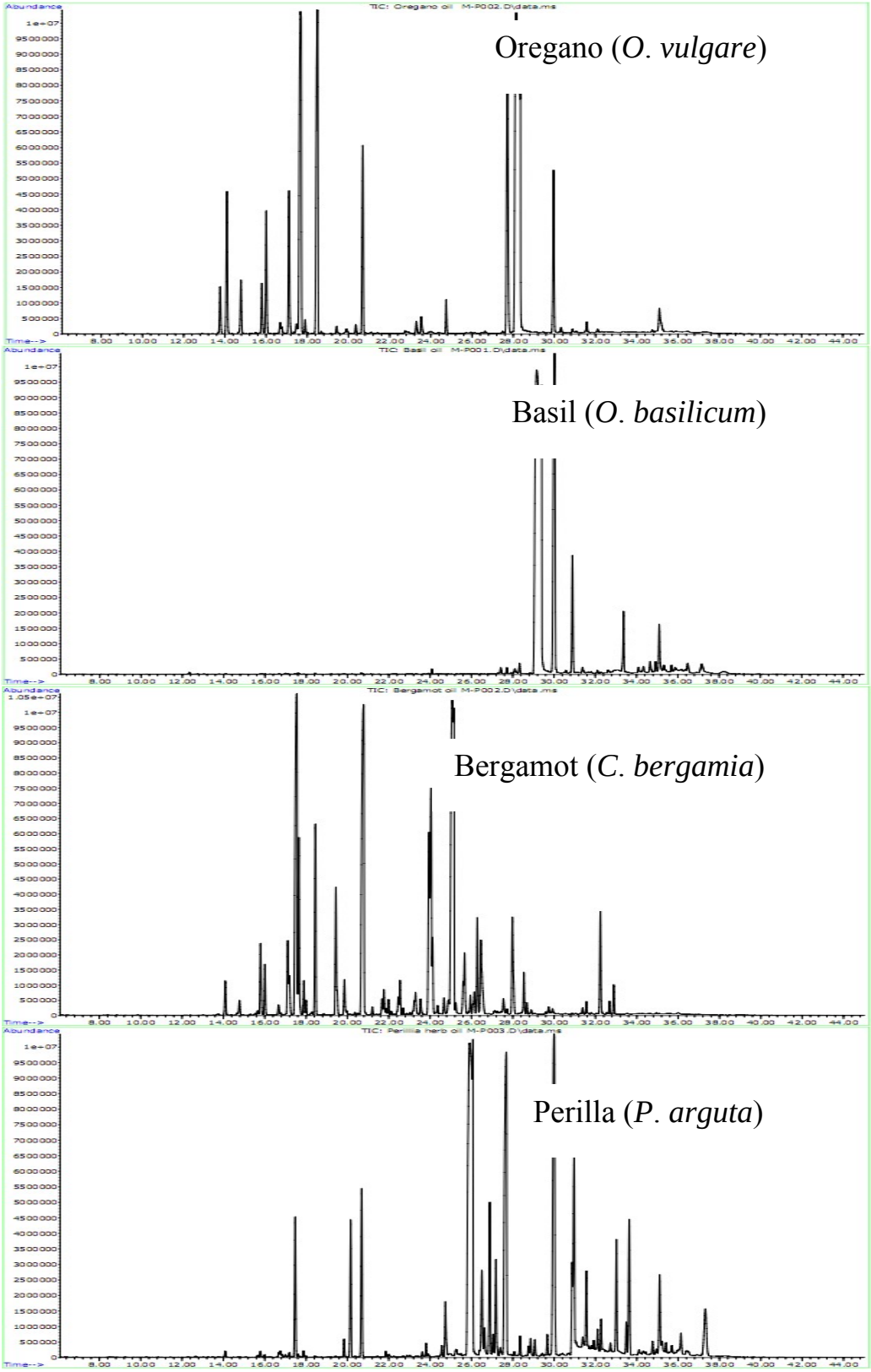

Fig.1. GC-MS chromatograms of four essential oils evaluated in this study as antimicrobial agents: oregano, basil, bergamot, and perilla.

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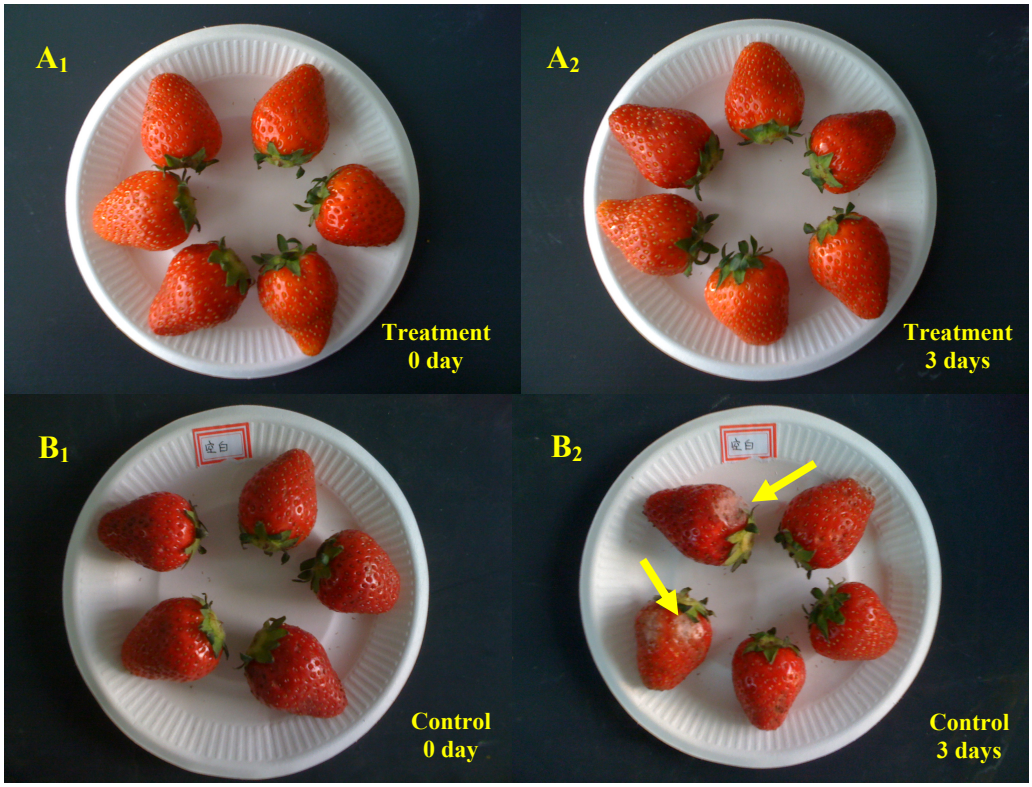

Fig.2. Photographs illustrate the preservative effects of the active preservative solution on strawberries: (A) treated group; (B) control group.

Table 1 Antimicrobial activity of investigated essential oils against the tested microorganisms.

samples		Inhibition zone diameter [a] (mm)			
		Gram$^+$		Gram$^-$	Yeast
		Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Saccharomyces cerevisiae*
EOs	Patchouli oil	10.1±0.4	10.4±0.3	nd	8.1±0.3
	Clary Sage oil	15.4±0.4	15.0±0.6	nd	10.0±0.4
	Rosemary oil	10.2±0.2	nd	8.6±0.2	12.3±0.5
	Basil oil	23.3±0.4	22.2±0.3	15.2±0.5	18.3±0.6
	Spearmint oil	nd	16.1±0.6	nd	12.6±0.8
	Oregano oil	27.4±0.5	27.4±0.7	18.2±0.8	27.2±0.6
	Perilla oil	nd	15.0±1.7	nd	25.9±0.2
	Absinthe oil	nd	15.2±0.6	nd	11.5±0.6
	Bergamot oil	26.2±0.6	24.3±0.6	nd	15.2±0.4
	Lavender oil	nd	13.5±1.0	nd	11.0±0.4
Antibiotic	KS	19.5±0.4	18.4±0.6	24.3±0.6	21.0±0.3
Chemical preservatives	SB	10.3±0.4	7.8±0.2	10.7±0.4	9.8±0.3

	PS	10.6±0.3	8.7±0.2	11.9±0.4	8.5±0.3
	ME	12.6±0.3	11.4±0.4	11.8±0.3	8.8±0.4
Sterile water		nd	nd	nd	nd

nd: not detected; The diameter of the discs (Ø = 6 mm) was included
KS: kanamycin sulfate (30 μg/disc); SB: sodium benzoate (200 μg/disc);
PS: potassium sorbate (200 μg/disc); ME: methylparaben (200 μg/disc).
[a] Results are presented as mean ± standard deviation from the experiments in triplicate.

Table 2 Minimal inhibitory concentration (MIC) (μl/ml) of selected essential oils against the tested microorganisms.

Microorganism		MIC (μl/ml)				
		basil	oregano	bergamot	perilla	KS
Gram$^+$	*Staphylococcus aureus*	1.25	0.625	1.25	5.0	0.3125
	Bacillus subtilis	0.625	0.625	1.25	2.5	0.3125
Gram$^-$	*Escherichia coli*	1.25	0.625	5.0	5.0	0.156
Yeast	*Saccharomyces cerevisiae*	2.5	0.625	2.5	0.625	0.3125

KS: kanamycin sulfate (50 μg/ml)**.**

Table 3. The main chemical compositions of essential oils of oregano, basil, bergamot and perilla.

NO.	RT (min)[a]	Compounds	GC area (%)[b]	RSD (%)[c]
Oregano (*O. vulgare*)				
1	17.675	p-cymene	15.10±0.05	0.36
2	18.487	γ-Terpinen	12.52±0.06	0.51
3	27.721	Thymol	8.79±0.03	0.35
4	28.145	Carvacrol	29.85±0.24	0.79
Basil (*O. basilicum*)				
1	29.165	Eugenol	62.97±0.12	0.19
2	30.083	Caryophyllene	21.57±0.12	0.56
Bergamot (*C. bergamia*)				
1	17.556	D-Limonene	13.35±0.01	0.10
2	20.782	Linalool	13.97±0.02	0.15
11	25.075	Bergamol	16.00±0.03	0.22
Perilla (P. *arguta*)				
1	25.918	2-Pentanoylfuran	23.03±0.04	0.19
2	27.689	Benzene,1-methoxy-4 -(1-methylpropyl)-	13.04±0.24	1.82

[a] RT = retention time (min)
[b] Relative proportions as percent of the total peak area, and results are presented as mean ± standard deviation from the experiments in triplicate
[c] Relative standard deviation (standard deviation divided by the mean).

Table 4 FIC indices (FICI) of complex essential oils against the tested microorganisms.

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Combinations	*Escherichia coli*		*Bacillus subtilis*		*Staphylococcus aureus*		*Saccharomyces cerevisiae*	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
1. Oregano-Basil	0.5 0.25	0.75 (ad)	0.5 0.25	0.75 (ad)	0.125 0.25	0.375 (S)	-	
2. Oregano-Bergamot	-		0.5 0.25	0.75 (ad)	0.25 0.125	0.375 (S)	-	
3. Basil-Bergamot	-		0.5 0.5	1 (ad)	0.25 0.125	0.375 (S)	-	
4. Oregano-Perilla	-		-		-		0.5 0.25	0.75 (ad)

-: not tested; S: synergism; ad: additive effect.

4. Conclusion

The antimicrobial activity of ten spice essential oils against four food-borne pathogenic microorganisms was evaluated and compared in this work. The essential oils displayed a variable degree of antimicrobial activity with oregano oil having the highest activity all the tested microorganisms. Basil oil and bergamot oil showed high effect against the Gram-positive bacteria (*S. aureus* and *B. subtilis*), while perilla oil exhibited a strong effectiveness against the yeast (*S. cerevisiae*). The four essential oils (oregano, basil, bergamot and perilla) selected for synergetic effect testing showed a large variation in their chemical composition. Oregano, basil and bergamot oils were dominated by phenols and terpenes, whereas the major active components of perilla oil were ketones. Combinations of spice EOs were assessed for synergistic activity and the results showed that a synergistic effect could be achieved for *S. aureus*. Consideration of the antimicrobial activity, organoleptic impact and cost, the complex EOs were selected as oregano-basil for *E. coli*, basil-bergamot for *S. aureus*, oregano-bergamot for *B. subtilis* and oregano-perilla for *S. cerevisiae*. The results of fresh-keeping test suggest that active preservative solution offers an attractive option for protecting food from microbial infestation and therefore have a potential for practical application in food industry.

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