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Synergistic interactions of cinnamaldehyde in combination with carvacrol against food-borne bacteria

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ABSTRACT

In order to select effective and safe natural antibacterial ingredients, more than 30 kinds of plant extracts were selected for their suitability as antibacterial agents. A standard broth microdilution method was used to evaluate their antimicrobial activity, a cytotoxicity test was used to detect their safety, and a synergy assay was used to determine which combinations have a synergistic effect. Then time-kill curves were used to further verify their bactericidal capacity. As a result of these tests, cinnamaldehyde and carvacrol were identified first, then subsequently verified to be the most effective and safe natural active substances. It was found that all of the tested bacteria strains were sensitive to cinnamaldehyde and carvacrol. The combination of cinnamaldehyde with carvacrol also showed good synergistic antibacterial effect against 7 of the 11 tested bacterial strains. The time-kill assay verified synergism for the cinnamaldehyde/carvacrol combination toward *Escherichia coli* and *Staphylococcus aureus*. These results indicated that the combination of cinnamaldehyde and carvacrol may serve as a promising naturally sourced food preservative with excellent bactericidal activity against common food spoilage microorganisms.

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1. Introduction

Food spoilage is a major problem that restricts the storage and transportation of food and also seriously impacts food safety. It drives us to find better ways to extend the shelf-life of foods. There are a lot of strategies to prevent pathogenic and spoilage microorganisms in food, and using chemical preservative is one of the important ways (Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012). However, there is currently a strong debate about the safety aspects of chemical preservatives, because they are considered to have many carcinogenic and teratogenic attributes as well as residual toxicity, consumers tend to be suspicious of chemical additives. For these reasons, alternative means are required (Hou, Shi, Zhai, & Le, 2007). At the same time, today's society appears to be experiencing a trend of 'green' consumerism. It means that food manufacturers should seek new 'green' or natural methods to make food safe. There is recent interest in the development of novel combinations of natural antimicrobial plant extracts to improve the quality and safety of agroindustrial products (Periago & Moezelaar, 2001; Periago, Palop, & Fernández, 2001).

Spices and their extracts are generally recognized as safe (GRAS), because of their traditional use without any documented detrimental impact (Newton, Lau, & Wright, 2000; Shan, Cai, Brooks, & Corke, 2007). Several of these spices and their essential oil extracts have been reported to posses antimicrobial activities. Cinnamon is traditionally harvested in Asian countries. And it has biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties. The principal constituents of cinnamon oil, namely cinnamaldehyde, exhibits an antimicrobial effect against a wide range of microorganisms (Sanla-Ead et al., 2012). In order to select effective and safe nature antibacterial ingredients, in this study, more than 30 kinds of plant extracts were selected as study objects. A standard broth microdilution method was used to detect their antimicrobial activity, cytotoxicity test was used to detect their safety and synergy assay were used to detect out which have synergistic effect. Then, time-kill curves were used to verify their bactericidal capacity deeply. This study will lay the foundation for the development of new type of the composite natural food preservatives.

2. Materials and methods

2.1. Reagents and strains

Thirty one kinds of plant extracts were selected as study objects, as presented in Table 1. All plant extracts were dissolved in

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Table 1
The Place of production, Purity and MIC values of 31 kinds of plant extracts.

Name	Place of production	Purity (%)	MIC (mg/mL) *E. coli*/*S. aureus*
Cinnamaldehyde	Aladdin Chemistry Co., Ltd. (Shanghai, China)	98.0	0.31/0.31
Curcumin	Chinese Institute of Food and Drug Test. (Beijing, China)	≥98.0	>2.50/>2.50
Citral	Baoji City, Best Plant Materials Co., Ltd. (Baoji, China)	97.0	2.50/2.50
P-Anisaldehyde	Chinese Institute of Food and Drug Test. (Beijing, China)	≥98.0	2.50/2.50
Allicin	Chinese Institute of Food and Drug Test. (Beijing, China)	88.4	>2.50/>2.50
Andrographolide	Chinese Institute of Food and Drug Test. (Beijing, China)	99.0	>2.50/>2.50
Eugenol	Chinese Institute of Food and Drug Test. (Beijing, China)	≥98.0	1.25/1.25
Carvacrol	Sigma–Aldrich. Inc. (St. Louis, USA)	98.0	0.31/0.31
Cinnamic acid	Chinese Institute of Food and Drug Test. (Beijing, China)	≥98.0	2.50/> 2.50
Citric acid	Chinese Institute of Food and Drug Test. (Beijing, China)	99.0	2.50/>2.50
Oleanolic acid	JiangXi Hengcheng Natural Spices Oil Co., Ltd. (Ji'an, China)	≥92.0	0.31/0.63
Perilla oil	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥98.0	≥2.50/2.50
Thymol	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	96.8	2.50/1.25
Clove oil	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	85.0	2.50/1.25
Peppermint oils	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	70.0	>2.50/>2.50
Naringin	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥95.0	0.31/0.63
Quercetin	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	98.0	0.31/0.63
Linalool	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥82.0	>2.50/>2.50
Geraniol	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	97.0	>2.50/>2.50
Camphor	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥97.0	0.63/0.31
Oregano oil	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥90.0	0.31/0.63
Dehydrocostus lactone	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥98.0	>2.50/>2.50
Usnic acid	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥98.0	2.50/>2.50
Chelerythrine	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥98.0	>2.50/>2.50
Resveratrol	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	98.0	2.50/>1.25
Zingerone	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	≥95.0	>2.50/>2.50
a-Terpineol	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	65.0–75.0	>2.50/>2.50
Sage oil	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	99.0	>2.50/>2.50
Artemisinin	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	99.0	>2.50/>2.50
Eugenyl acetate	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	98.0	1.25/0.63
Apigenin	Jiangxi Kangsheng Tang Pharmaceutical Co., Ltd. (Ji'an, China)	98.0	>2.50/2.50

dimethyl sulfoxide (DMSO) at a concentration of 5 g/L under sterile conditions and stored at 4 °C when use. The microbial strains were obtained from the Institute of Zoonosis in Jilin University (Changchun, P.R. China). All the selected strains include the Gram-positive bacteria and the Gram-negative bacteria, as shown in Table 2.

2.2. MIC determination

The resistance of bacteria to the antibiotics and the minimum inhibitory concentration (MIC) of natural antimicrobials were determined by standard broth microdilution (Wiegand, Hilpert, & Hancock, 2008). Beef-extract peptone medium was used for the food-borne bacteria. Active cultures were generated by inoculating the thawed microbial stock suspensions into nutrient broth followed by overnight incubation at 37 °C with shaking. The inoculums were adjusted to final concentrations of 1×10^5 cfu/mL by comparison with a McFarland No. 1 turbidity standard. The natural antimicrobials were dissolved in beef-extract peptone medium with DMSO in two-fold serial dilutions from 5 mg/mL to 0.078 mg/mL. Then, 100 μl of the individual antimicrobials and 100 μl of the 1×10^5 cfu/mL bacteria were dispensed into the 96 well plates. Beef-extract peptone medium blank was used as a control. The MIC was defined as the lowest antibiotic or antimicrobial concentration which prevented visible growth (Fazeli et al., 2007). After 24 h incubation at 37 °C, 10 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) which dissolved in phosphate buffer solution (PBS) at a concentration of 5 mg/mL was added to each well, and then incubated for 4 h at 37 °C. MTT, which is a yellow tetrazole, is taken up into cells by endocytosis or protein-facilitated mechanism and reduced, mainly by mitochondrial enzymes, to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation within living cells. While the dead cells don't have this feature. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability. Which can be detected by the color using the naked eye. The MIC was the lowest agent concentration at which no purple color (loss of metabolic activity) appeared. All tests were performed in triplicate.

Table 2
MIC values of Cin and Car against 11 food-borne bacteria.

Bacteria	MIC (mg/mL)		Bacteria	MIC (mg/mL)	
	Cin	Car		Cin	Car
E. coli	0.31	0.31	*S. sanguinis*	0.63	0.16
S. aureus	0.31	0.31	*E. cloacae*	0.63	0.16
Y. regensburgei	0.31	0.31	*S. haemolyticus*	0.16	0.31
S. intermedius	0.16	0.31	*A. hydrophila*	0.31	0.16
K. kristinae	0.31	0.31	*S. enteritidis*	0.31	0.31
L. garvieae	0.63	0.16			

2.3. Synergy testing

The checkerboard method used for measurement of interactive inhibition (Pillai, Moellering, & Eliopoulos, 2005) was determined for synergy between the cinnamaldehyde and carvacrol. In this study, the antimicrobial agents were dissolved in beef-extract peptone medium with DMSO to obtain final concentrations that ranged from five dilutions below the 1/2 MIC value obtained from the MIC determination using two-fold dilutions. The effects of combinations were evaluated by calculating the fractional inhibitory concentration (FIC) index which was combined with the checkerboard method (Pei, Zhou, Ji, & Xu, 2009) for each combination using the following formula. The FIC was used to interpret the test results as follows: FIC $\leq$ 0.5, synergy; FIC = 0.5–4, no

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