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Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food

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Carvacrol and cymene, phenolic compounds naturally present in the essential oil of oregano and thyme, were examined for their antimicrobial activity against *Vibrio cholerae* (ATCC 14033, VC1, and VC7) inoculated in carrot juice. Carvacrol exhibited a dose dependent inhibitory effect on the bacteria. Although cymene did not have antimicrobial activity against the bacteria, it enhanced the inhibitory ability of carvacrol. At 25 °C, the lowest concentrations of carvacrol and cymene required for zero detectable viable count varied depending on bacterial strains; 5 and 5 ppm, respectively, for VC7; 5 and 7.5 ppm, respectively, for VC1; and 7.5 and 7.5 ppm, respectively, for ATCC 14033. This study also examined several factors influencing the antimicrobial activity of carvacrol and cymene against *V. cholerae* ATCC 14033, including temperature, bacterial cell number, and food substrate. Carvacrol and cymene inhibited the bacterium in carrot juice at 25 °C more efficiently than at 15 and 4 °C. The doses of both compounds required for zero detectable viable count increased as the number of the bacterial cells in the carrot juice increased. The fat content and the complexity of foods were shown to decrease the antimicrobial activity of the compounds.

[**Key words:** Antimicrobial activity; Carvacrol; Cymene; *Vibrio cholerae*]

Vibrio cholerae is a comma-shaped Gram-negative bacterium. It is motile with a polar flagellum. Catalase and oxidase positive cells are facultatively anaerobic and capable of both fermentative and respiratory metabolism. *V. cholera* can be subdivided into serotypes based on somatic O antigens. Although most pathogens belong to the O1 serotype, members in the O139 serotype are also known to be causative agents of cholera (1). The O139 serotype was associated with epidemic cholera in India and Bangladesh and has also been isolated from cholera patients in Thailand (2,3). The O141 serotype has recently emerged in the United States and has the potential to cause outbreaks of a cholerae-like illness (4,5). Although the epidemiology of *V. cholera* O141 is not well-understood, consumption of foods contaminated with the bacterium appears to be a possible vehicle for foodborne transmission.

Cholera is an acute infection of the gastrointestinal tract caused by *V. cholerae*. The disease remains endemic in many developing countries, particularly in southern Asia, Africa, and Latin America. The severe, watery non-bloody diarrhea, known as rice water stool because of its appearance, can result in the loss of a liter of fluid per hour, and it is this fluid loss and the consequent electrolyte imbalance that results in marked dehydration, metabolic acidosis, hypokalemia, and hypovolemic shock resulting in cardiac failure. Untreated, mortality from cholera is approximately 40–60%. Fluid and electrolyte replacement, instituted rapidly, reduces mortality to less than 1% (1). Cholera is regarded primarily as a waterborne infection, though food that has been in contact with contaminated water can often serve as a vehicle. For example, a large number of different foods have been implicated in outbreaks, particularly products which are consumed without cooking (6). The infectious dose in normal healthy individuals is large when the organism is ingested without food, of the order of 10^{10} cells, but it is considerably reduced if consumed with food, which protects the bacterium from stomach acidity. Studies conducted in Bangladesh indicate that 10^3 to 10^4 cells may be a more typical infectious dose (1).

Some food preservation systems, such as heat treatments and addition of synthetic preservatives, can be used to reduce risk of outbreaks of *V. cholera* infection. However, these systems can have undesired effects and are contrary to food industries' and consumers' demands, who ask for additive-free, fresher, and more natural-tasting food products while maintaining microbiological safety (7,8). It has been suggested that many natural antimicrobial compounds from plant, animal, and microbial sources might fulfill this demand (7,9,10).

Herbs have been known for their antimicrobial activity since ancient times. The safe use of herbs and their components has led to their current status of generally recognized as safe (GRAS) food ingredients (10,11). Carvacrol and cymene (a precursor of carvacrol) are among natural compounds having potential to be used as preservative agents. They are phenolic compounds present in the essential oil fraction of oregano and thyme (11). Carvacrol has been found to exhibit antimicrobial activity against a variety of bacteria, including foodborne pathogens (12–14). Although cymene does not inhibit the growth of bacteria (12,15,16), it can enhance the

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antimicrobial activity of carvacrol (17). Studies on the potential use of carvacrol and cymene to control foodborne pathogens in foods have been carried out with *Bacillus cereus* (13) and *Escherichia coli* O157:H7 (18). However, no such study has been performed with *V. cholera*. Therefore, in the present study, the inhibitory effect of carvacrol and cymene alone and combined against *V. cholera* artificially contaminated in foods was investigated. Several factors that may influence the effect, including bacterial strain, temperature, bacterial cell number, and food substrate, were also evaluated.

MATERIALS AND METHODS

Bacterial strains and culture conditions One reference strain (ATCC 14033) and 2 clinical strains (VC1 and VC7) of *V. cholerae* were included in the study. The reference strain (serotype O1) was obtained from the American Type Culture Collection (ATCC). The VC1 (serotype O1) and VC7 (serotype O139) were isolated from patients suffering from cholera in Tak Province, Thailand. The identity of these strains was confirmed by a variety of biochemical, physiological, and serological tests, as described previously (19). All the bacteria used in this study were grown at 37 °C in BHI (Brain Heart Infusion) broth. Bacterial stock cultures were stored as frozen cultures at −80 °C in BHI broth containing 20% glycerol (v/v).

For the preparation of bacterial cells to inoculate food samples, fresh overnight cultures of *V. cholerae* in BHI broth were washed three times and resuspended in sterile saline solution. Viable cell counts of the washed cell suspensions were determined by serial dilution (1:10) in sterile peptone water, spreading 0.1 ml of the suspensions (in triplicate) on TCBS agar, a selective medium for *Vibrio*, and incubating the plates at 37 °C for 24 h. One liter of TCBS agar contained 10 g of peptone, 5 g of yeast extract, 10 g of sodium citrate, 10 g of sodium thiosulfate, 5 g of ox bile, 3 g of sodium cholate, 20 g of sucrose, 10 g of sodium chloride, 1 g of iron (III) citrate, 0.04 g of thymol blue, 0.04 g of bromothymol blue, and 14 g of agar.

Chemicals Carvacrol and cymene were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Solutions of these compounds were prepared in sterile distilled water.

Foods Foods used in this study were carrot juice, fish broth, and vegetable soup. Freshly pressed, raw juice from carrots was obtained directly from a manufacturer in Ubon Ratchathani Province, Thailand. The juice was heated with steam for 15 min, cooled down, and stored at 4 °C. The final pH was 6.7, and the fat content was less than 0.01%. Just before the experiments, the juice was taken out from the refrigerator, and its temperature was adjusted to 25 °C. Commercial fish broth was obtained from a local retail store and stored at 4 °C. The pH was 6.5 and the fat content was 0.5%. The product was heated with steam for 15 min and then cooled to 25 °C before being inoculated. Vegetable soup consisted of chicken broth with diced onion, celery, carrot, and potato. The amount of vegetable in the soup was 20% (w/v). It was obtained from a local retail store and stored at 4 °C. The pH was 6.8 and the fat content was 0.47%. The product was heated with steam for 15 min and then cooled to 25 °C before being inoculated.

Microbiological analysis Ten milliliters of food samples were taken and subjected to serial (1:10) dilutions in sterile peptone water. Appropriate dilutions of the samples were spread on TCBS agar, a selective medium for *Vibrio*. Bacterial colonies grown on the agar were confirmed to be *V. cholerae* by the method described above. The numbers of the bacterial cells grown on TCBS agar were used to calculate the concentration of the bacteria in the foods. Single samples from each duplicate batch of the foods were removed periodically during incubation for counting viable cells and plating out in triplicate; therefore, mean counts for each time point were calculated from six replicate determinations.

Antimicrobial activity of carvacrol and cymene againts *v. cholerae* in food Carrot juice was prepared in duplicate for each treatment. Carvacrol and cymene were added to the juice alone or in combination to give final concentrations as shown in Table 1. The juice was inoculated with the bacterial suspensions (prepared as described above) to give an approximate count of 10^5 CFU/ml. Control juices were inoculated with the bacteria but without carvacrol and cymene. Samples were taken for determination of viable cell counts after storing for 4 days at 25 °C.

Effect of temperature on antimicrobail activity of carvacrol and cymene Carrot juice was prepared to contain 7.5 ppm of carvacrol, 7.5 ppm of cymene, and 10^5 CFU/ml of *V. cholerae* ATCC 14033. The juice inoculated with the same amount of the bacterial strain, but not with carvacrol and cymene, was used as a control. The tested and control juices were divided into 3 groups for storage at 3 different temperatures, 4, 15, and 25 °C. Viability of the bacterial strain was examined in the samples taken from each group at the time of bacterial inoculation and every 12 h for 4 days post-inoculation. This experiment was performed in duplicate.

Sensitivity to carvacrol and cymene of *V. cholerae* ATCC 14033 in carrot at different storage temperature was determined by using various combinations of the compounds. The concentrations of carvacrol and cymene included in this experiment were 7.5, 10, 12.5 and 15 ppm. Carrot juice (in duplicate) treated with different combinations of carvacrol and cymene was inoculated with *V. cholerae* ATCC 14033 (10^5 CFU/ml) and stored at 4 and 15 °C. Viable counts were determined in samples taken after storage for 1 day.

TABLE 1. Viable count (mean ± SD) (log CFU/ml) of *V. cholerae* in carrot juice after 4 day storage at 25 °C in the presence of different combinations of carvacrol and cymene.

V. cholerae	Concentration of carvacrol (ppm)	Concentration of cymene (ppm)			
		0	2.5	5	7.5
ATCC 14033	0	6.21 ± 0.19	6.19 ± 0.15	6.16 ± 0.20	6.20 ± 0.13
	2.5	4.62 ± 0.08	4.23 ± 0.18	3.75 ± 0.16	3.11 ± 0.07
	5	4.47 ± 0.07	3.96 ± 0.11	3.28 ± 0.12	2.14 ± 0.09
	7.5	3.75 ± 0.05	3.22 ± 0.03	2.02 ± 0.07	Undetectable
VC1	0	6.21 ± 0.14	6.23 ± 0.11	6.19 ± 0.15	6.23 ± 0.10
	2.5	4.45 ± 0.13	3.96 ± 0.06	3.24 ± 0.09	2.51 ± 0.08
	5	4.02 ± 0.09	3.35 ± 0.14	2.37 ± 0.08	1.83 ± 0.04
	7.5	3.28 ± 0.07	2.51 ± 0.06	Undetectable	Undetectable
VC7	0	6.22 ± 0.09	6.17 ± 0.05	6.20 ± 0.06	6.18 ± 0.11
	2.5	4.22 ± 0.08	3.67 ± 0.10	2.53 ± 0.07	1.46 ± 0.04
	5	3.38 ± 0.12	2.44 ± 0.06	Undetectable	Undetectable
	7.5	2.58 ± 0.07	1.79 ± 0.04	Undetectable	Undetectable

Effect of bacterial cell number on antimicrobial activity of carvacrol and cymene Carrot juice (in duplicate) was inoculated with *V. cholerae* ATCC 14033 to obtain the final concentration of 10^3, 10^5, or 10^7 CFU/ml. To the treated juice carvacrol (7.5 ppm) and cymene (7.5 ppm) were added simultaneously. Controls consisted of the juice inoculated with the bacterial strain without carvacrol and cymene. Inoculated juice was stored at 25 °C and enumerated for *V. cholerae* at the time of bacterial inoculation and every 12 h for 4 days during the storage period.

To determine the concentrations of carvacrol and cymene required for complete inhibition (no detectable viable cell) of 10^7 CFU/ml of *V. cholerae* ATCC 14033 in carrot juice, different combinations of both compounds were added to the inoculated carrot juice. The concentrations of carvacrol and cymene included in this experiment were 7.5, 10, 12.5, and 15 ppm. Viable counts were determined in samples taken after 4 days of storage at 25 °C.

Effect of food substrate on antimicrobial activity of carvacrol and cymene In this experiment, 3 different types of food were used including carrot juice, fish broth, and vegetable soup. All types of the food were identically treated with carvacrol (7.5 ppm), cymene (7.5 ppm), and *V. cholerae* ATCC 14033 (10^5 CFU/ml). Inoculated foods without carvacrol and cymene served as controls. All of the treated foods were stored at 25 °C. Viable counts of *V. cholerae* were examined in the samples taken at the time of bacterial inoculation and every 12 h for 4 days of storage. Each treatment was performed in duplicate.

To determine the concentrations of carvacrol and cymene required for complete inhibition of *V. cholerae* ATCC 14033 in fish broth and vegetable soup, different combinations of both compounds were added to the foods inoculated with *V. cholerae* to gain the final concentrations of 10^5 CFU/ml. The concentrations of carvacrol and cymene included in this experiment were 7.5, 10, 12.5, and 15 ppm. Viable counts were determined in samples taken after 4 days of storage at 25 °C.

RESULTS

Antimicrobial activity of carvacrol and cymene against *V. cholerae* strains was studied in carrot juice at 25 °C by determining the viable cell counts 4 days after treatment. In control (no carvacrol and cymene) groups, total viable cell counts increased from 5 log CFU/ml to 6.2 log CFU/ml (Table 1). The results indicated that all tested strains of *V. cholerae* survived and grew similarly in the juice at 25 °C. Carvacrol, when used separately, exhibited a dose dependent inhibitory effect on all strains of *V. cholerae*, though to different degrees. These were indicated by the reduction of the viable counts of the bacteria as the concentrations of carvacrol increased (Table 1). In contrast, cymene, when used alone, had no antimicrobial activity against all test strains. The viable bacterial cells determined in carrot juice treated with different concentration of cymene were very similar to those obtained from the control groups (Table 1). Although cymene lacked antimicrobial activity against *V. cholerae*, it enhanced the inhibitory effect of carvacrol. The antimicrobial activity of carvacrol when used together with cymene was dependent on dose of cymene and strain of bacteria. Regardless of bacterial strain the antimicrobial activity of carvacrol increased as the concentration of cymene increased. Of all test bacteria, *V. cholerae* VC7 was the most sensitive strain to carvacrol and cymene, whereas *V. cholerae* ATCC 14033 was