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Short Communication

In vitro study of synergistic antimicrobial effect of carvacrol and cymene on drug resistant *Salmonella typhi*

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The ineffective use of antibiotics in inhibiting drug resistant *Salmonella typhi* has lead to the search of alternative compounds to replace antibiotics. In this study, carvacrol and cymene, compounds naturally present in oregano, thyme and savory were tested for their antimicrobial activity against 4 strains of drug resistant *S. typhi*, STC1 to STC4. Carvacrol, but not cymene was found to be able to inhibit all test strains. STC4 and STC1 were the most and the least sensitive strains with the minimal inhibitory concentration (MIC) of 13.02 and 62.50 µg/ml, respectively. Synergism between carvacrol and cymene against drug resistant *S. typhi* was observed. The MICs of carvacrol against all test strains decreased when used together with cymene (5 and 10 µg/ml). Carvacrol exhibited a bactericidal effect of carvacrol on the sensitive cells both in the presence and the absence of cymene.

Key words: Antimicrobial activity, carvacrol, cymene, *Salmonella*.

INTRODUCTION

Salmonella typhi is a gram-negative enteric bacillus belonging to the family Enterobacteriaceae. It is a non-spore forming, motile, facultative anaerobe. *S. typhi* is a causative agent of typhoid or enteric fever which is characterized by the sudden onset of a sustained and systemic fever, severe headache, nausea, diarrhea and loss of appetite. The mortality rate of untreated cases ranges from 12 to 30% while that of treated cases is about 1%. The disease is still a common disease in parts of the world with poor sanitation including countries in South America, African and Southeast Asia (Murray, 1994).

Treatment of typhoid based on antibiotics is now questionable. It may lead to the development of drug resistant bacteria which can transfer the undesired trait to environment and other human pathogenic bacteria (Gould, 1999). Many drug resistant strains of *S. typhi* have been isolated and found to be associated with numerous outbreaks of typhoid fever in many countries in the Indian subcontinent, Southeast Asia and Africa (Rowe et al., 1997). Since antibiotic based approach is ineffective in controlling typhoid fever caused by drug resistant *S. typhi*, it is of importance to search for an alternative approach to treat the disease. One approach that may be useful for controlling drug resistant *S. typhi* infection is to use natural compounds generally recognized as safe (GRAS) to inhibit the bacterium. Plant derived compounds are among the potential candidates for such purpose because many of them have been reported to have antimicrobial activity (Cowen, 1999).

Carvacrol is a biological compound naturally present in plants such as oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and savory (*Satureja hortensis*). It has been shown to have a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria (Friedman et al., 2002). Its potential to inhibit bacteria was also reported in many foods such as rice, apple juice, kiwifruit, honeydew melon and semi skimmed milk (Burt, 2004). Cymene, a biological precursor of carvacol, by itself does not have antimicrobial activity but it can enhance the inhibitory effect of carvacrol when used together (Burt, 2004). However, antimicrobial activity of carvacrol and cymene against drug resistant *S. typhi* has never been reported. In this study, carvacol and cymene were examined *in vitro* for their antimicrobial activity against drug resistant *S. typhi*. Synergistic antimicrobial effect between both compounds was also studied.

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Table 1. Antimicrobial activity of carvacrol and/or cymene against drug resistant *S. typhi*.

S. typhi	Inhibition zones (mm)[a]		
	Carvacrol	Cymene	Carvacrol and cymene
STC1	12.8 ± 0.4	0	17.6 ± 0.8
STC2	17.3 ± 0.6	0	22.3 ± 0.6
STC3	15.6 ± 0.3	0	19.7 ± 0.7
STC4	20.4 ± 0.4	0	25.2 ± 0.7

[a]Results are mean ± S.D. values of three replicates.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Four drug resistant strains of *S. typhi* used in this study including STC1, STC2, STC3 and STC4 were clinical strains isolated from stools of typhoid fever patients. They were resistant to chloramphenicol, ampicillin, sulfomehoxazole, trimethoprim, gentamicin and cephaloridine. All of them were kindly donated by Sunprasitthiprasong Hospital, Ubon Ratchathani Province, Thailand. The identity of the bacterial strains was confirmed using the API 20 E test kit (bioMerieux Industry, Hazelwood, MO, USA). Brain heart infusion (BHI) medium (Oxoid GmbH, Wesel, Germany) was used to culture the bacterial strains at 37ºC. The bacterial stock cultures were stored as a frozen culture at -80ºC in BHI broth containing 20% glycerol (vol/vol).

Chemicals

Carvacol and cymene were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Solutions of these compounds were prepared in 95% ethanol.

Antimicrobial activity testing

The antimicrobial activity of carvacrol and/or cymene against drug resistant *S. typhi* was evaluated by the swab paper disc method (Rattanachaikunsopon and Phumkhachorn, 1998) with some modifications. The bacterial culture (10^5 CFU/ml) was spread with a sterile swab on BHI agar, before placing sterile filter paper discs of 6 mm in diameter (Schleicher and Schuell, USA) on the agar. 30 µl of each test solution were spotted on the paper disc. Tested solutions used in this study were carvacrol solution (1 mg/ml), cymene solution (1 mg/ml), carvacrol/cymene solution (1 mg/ml of each compound). Oxytetracycline (5 µg/disc) was used as a positive control, while 95% ethanol was used as a negative control. After incubating at 37ºC for 24 h, the diameter of each growth-free zone around the discs was measured and the diameter of the paper disc subtracted, giving the size of each inhibition zone beyond the paper disc. Studies were performed in triplicate.

Determination of minimal inhibitory concentration (MIC)

The MIC value of carvacrol for *S. typhi* was determined by using the microtiter broth microdilution method described by Amsterdam (1996) with some modifications. The experiment was performed in a microtiter plate. Each well of the microtiter plate contained 200 µl of BHI broth with *S. typhi* cells at a final concentration of 10^3 CFU/ml. The concentration of carvacrol in the plate ranged from 500 to 0.12 µg/ml. Positive and negative controls were included. Positive control was the *S. typhi* culture at the concentration of 10^4 CFU/ml without cravacrol while negative control was BHI broth without the test bacterium and compound. After incubation at 25°C, bacterial growth was determined at 24 h by measuring absorbance at 600 nm using the EL x 800 universal microplate reader (Biotek Instrument Inc., Highland Park, Verment, USA). The MIC of carvacrol was defined as the lowest concentration causing the decrease of the absorbance when compared with the positive control. Three replicates were performed for each concentration of the compounds. Other two sets of experiment were performed to determine the MIC value of carvacrol plus cymen for *S. typhi*. The experiments were conducted the same as mentioned above except adding 5 and 10 µg/ml of cymene to microtiter plates.

Examination of effect on *S. typhi* cells

Carvacrol or carvacrol plus cymene with a final concentration equals to the MIC value was added to 4.9 ml of *S. typhi* culture (10^4 CFU/ml). After incubation at 37°C for 24 h, 100 µl of the mixtures were inoculated into 4.9 ml of fresh BHI. For a control, the same volume of untreated culture of *S. typhi* with a concentration of 10^4 CFU/ml was transferred to 4.9 ml of fresh BHI. Bacterial growth of the treatment and control cultures was determined after incubation at 37°C for 24 h by measuring the optical density at 600 nm. The experiments were done in three replicates.

RESULTS AND DISCUSSION

Antimicrobial activity of carvacrol, cymene and carvacrol plus cymene against four different drug resistant strains of *S. typhi* was qualitatively determined by swab paper disc method. Carvacrol was found to be able to inhibit growth of the bacteria with different degree of inhibition. Based on size of inhibition zone, *S. typhi* strain STC4 was the most sensitive strain with the diameter of about 20.4 mm whereas *S. typhi* strain STC1 was the least sensitive with the diameter of about 12.8 (Table 1). On the other hand, cymene did not inhibit all of the tested bacteria. Regardless of bacterial strain, antimicrobial activity of carvacrol increased when used together with cymene, indicated by the increase in the size of inhibition zones. These results suggested that cymene enhanced the antimicrobial activity of carvacrol. When carvacrol and cymene were used together, the most and the least sensitive strains were still STC4 and STC1.

When carvacrol was subjected to the determination of MIC values against all of the tested *S. typhi* strains, there is in general a good agreement between the findings for the swab paper disc method and the MIC determination assay. The MIC values varied depending on the strain of *S. typhi*. STC4 and STC1 gave the lowest (13.03 µg/ml) and highest (62.50 µg/ml) MIC values, respectively (Table 2). These results indicated that STC4 and STC1 were the most and the least sensitive strains, respectively. The synergistic antimicrobial effect between carvacrol and cymene was also evident. The MICs of carvacrol deceased when used together with cymene. The reduction of the MICs was dependent on the concentration of cymene. For example, the MIC of carvacrol

Table 2. MIC values of carvacrol against drug resistant *S. typhi* when used alone and used together with 0.5 and 10 µg/ml of cymene.

S. typhi	MIC (µg/ml) [a]		
	Carvacrol	Carvacrol with cymene (5 µg/ml)	Carvacrol with cymene (10 µg/ml)
STC1	62.50 ± 0.00	20.84 ± 7.36	6.51 ± 1.84
STC2	31.25 ± 0.00	10.42 ± 3.69	3.91 ± 0.00
STC3	26.04 ± 7.36	15.63 ± 0.00	3.25 ± 0.92
STC4	13.02 ± 3.69	5.21 ± 1.83	1.63 ± 0.46

[a]Results are mean ± S.D. values of three replicates

against STC1 was reduced from 62.50 µg/ml when used alone to 20.84 and 6.51 µg/ml when used in conjunction with 5 and 10 µg/ml of cymene, respectively (Table 2). This reduction pattern of MIC was also found in the other tested strains of *S. typhi*.

To study effect of carvacrol on the various *S. typhi* strains used in this study, recovery of *S. typhi* inhibited by the compound for 24 h was examined in fresh BHI broth. It was found that the bacteria inhibited by carvacrol did not resume growth in the fresh BHI broth within 24 h. On the other hand, the untreated (control) *S. typhi* cells grew in the fresh BHI broth. These results suggested that carvacrol had a bacteriocidal effect on all test *S. typhi.* The effect of carvacrol on *S. typhi* cells did not affect by cymene. This indicated by the experiments that performed the same as above except using carvacrol together with 5 and 10 µg/ml of cymene instead of carvacrol alone. The results showed that bacteria inhibited by carvacrol together with cymene did not resume growth in the fresh BHI broth within 24 h.

Mechanism of action of carvacrol and cymene which has been extensively studied may help to explain the synergism between both compounds in some extent. Carvacol destabilizes the cytoplasmic membrane and in addition, acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. The resulting collapse of the proton motive force and depletion of the ATP pool eventually lead to cell death (Ultee et al., 2002). Cymene, a precursor of carvacrol which lacks a hydroxyl group, was found to have a higher preference for liposomal membranes, thereby causing swelling of the cytoplasmic membrane to a greater extent than does carvacrol (Ultee et al., 2002). Because it does not affect pH gradient and the ATP pool, it is ineffective in killing cells when used alone. When carvacrol and cymene are used together, a synergistic effect between both compounds happens. It may be due to the fact that cymene accumulating in and causing the expansion of the plasma membrane enables carvacrol to be more easily transported into the cell.

This is the first study presenting the successful use of carvacrol and carvacrol in combination with cymene in inhibiting drug resistant *S. typhi in vitro*. These results may be useful in medicine and food industry to develop effective approaches to control drug resistant *S. typhi* infecting patients and contaminated in food, respectively.

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