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Inhibitory activity of Thai condiments on pandemic strain of *Vibrio parahaemolyticus*

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Abstract

Antibacterial activity of 13 condiments used in Thai cooking was investigated with a pandemic strain of *Vibrio parahaemolyticus*. Using a disk diffusion technique, freshly squeezed extracts from galangal, garlic and lemon, at a concentration of 10 µl/disk produced a clear zone of 13.6±0.5, 11.6±0.5 and 8.6±1.2 mm, respectively. The inhibitory activity of these 3 condiments on pandemic strains was not significantly different from that on non-pandemic strains of *V. parahaemolyticus*. Because of its popularity in seafood cooking, galangal was subjected to further investigation. Only a chloroform extract of galangal inhibited growth of *V. parahaemolyticus* producing a clear zone of 9.5±0.5, 12.0±0 and 13.5±0.5 mm diameter at concentrations of 25, 50 and 100 µg/disk, respectively. One active component is identified as 1'-acetoxychavicol acetate. The activity of galangal was not reduced at pH 3 or in the presence of 0.15% bile salt but was reduced by freeze and spray drying. Heating a fresh preparation of galangal to 100 °C but not 50 °C for 30 min also reduced growth inhibition. Therefore, using fresh galangal in cooking was recommended. The MIC and MBC of a freshly squeezed preparation of galangal were 1:16 and 1:16, respectively. This is the first report of an inhibitory activity of a Thai medicinal plant, galangal that is used in Thai cooking, on the pandemic strain of *V. parahaemolyticus*.

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1. Introduction

Vibrio parahaemolyticus is a halophilic Gram-negative bacterium that causes acute gastroenteritis in humans. Food-poisoning caused by this pathogen is generally associated with the consumption of raw or undercooked seafood. Clinical manifestations include diarrhea, abdominal cramps, nausea, vomiting, headache, fever and chills, with incubation periods of from 4 to 96 h. Most clinical strains of *V. parahaemolyticus* possess a major virulence factor, the thermostable direct hemolysin (TDH) which exhibit β-hemolysis on Wagatsuma agar (Miyamoto et al., 1969; Sakurai et al., 1973; Nishibuchi and Kaper, 1995). Another virulence factor, TDH-related hemolysin (TRH) has also been involved in some food-poisoning outbreaks

(Honda et al., 1988). TDH and TRH are encoded by the *tdh* and *trh* genes. Serotypes of *V. parahaemolyticus* can be differentiated into 13 O groups and 71 K types (Iguchi et al., 1995). A predominant new strain belonging to serovar O3:K6 appeared for the first time in February 1996 in Calcutta, India and later accounted for 50–80% of the strains isolated from clinical specimens taken in India between February to August 1996 (Okuda et al., 1997). Since then, the new O3:K6 strains have been considered to be the first pandemic strains of *V. parahaemolyticus*, and are involved in a high proportion of foodborne poisoning outbreaks in the United States (Center for Disease Control and Prevention (CDC), 1998, 1999) and several Asian countries including Thailand (Aragawa et al., 1999; Chiou et al., 2000; Matsumoto et al., 2000; Vuddhakul et al., 2000). In addition, O4:K68, O1:KUT and O1:K25 have been reported as pandemic strains and originate from the same clone as O3:K6 (Matsumoto et al., 2000;

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Laohaprertthisan et al., 2003). These pandemic strains express the *tdh* gene and possess the ability to adapt to differing environmental conditions. In addition, the adherence and cytotoxicity of these strains are superior to other serotypes (Yeung et al., 2002). In Thailand, the incidence of *V. parahaemolyticus* infections in Hat-yai hospital, southern Thailand has been reported. During the years 2000 and 2001, 125 cases out of 187 cases (66.4%) were due to infections by pandemic strains. Dominance of the O3:K6 followed by the O1:K25 serotype was observed among these pandemic strains. Seafood especially shellfish was investigated during the same period in Hat-Yai city; 12 isolates of pandemic strains of *V. parahaemolyticus* were isolated from molluscan shellfish, *Perna viridis*, *Meretrix meretrix* and *Anadara granosa*. A comparison of DNA fingerprints of strains isolates from the shellfish and clinical strains showed identical patterns, indicating that these pandemic strains have contaminated the environment. Therefore, shellfish and other marine organisms are the major sources of infection (Vuddhakul et al., accepted).

Emergence of a pandemic strain of *V. parahaemolyticus* has triggered immense interest in the search for active compounds from Thai food ingredients to protect against this organism, as traditional Thai seafood dishes often use spices and herbs for aroma and flavor. Some of these ingredients, including garlic, onion, turmeric, galangal and krachai, have been reported to have antimicrobial activity (Vohora et al., 1973; Fransworth and Bunyapraphatsara, 1992; Apisariyakul et al., 1995; Naganawa et al., 1996; Sawangjaroen et al., 2004). Therefore, in this study, we attempted to investigate the inhibitory activity of Thai condiments on a pandemic strain of *V. parahaemolyticus*.

2. Materials and Methods

2.1. Bacterial strains

Two clinical isolates of pandemic strains of *V. parahaemolyticus* O3:K6 and O1:K25 designated as PSU 335 (*tdh+ve*), PSU 414 (*tdh+ve*) and two clinical isolates of non-pandemic strains O3:K6 and O3:K56 designated as PSU 253 (*tdh-ve*) and PSU 1702 (*tdh+ve* and *trh+ve*) from Hat-yai hospital were used in this study. Standard strains of *Escherichia coli* ATCC 29522 and *Staphylococcus aureus* ATCC 29523 were used as controls. They were grown for 4 h in Muller Hinton broth (MHB) containing 1% NaCl at 37°C and adjusted to 1.5×10^8 cells/ml by turbidimeter (Oxoid, England).

2.2. Freshly squeezed extracts

Thirteen popular condiments used in Thai spicy soup and spicy salad were investigated (Table 1). They were cut and blended in a blender (Toshiba), then they were squeezed by a garlic crusher. Aqueous solution was

Table 1
Inhibitory activity of freshly squeezed extract from 13 Thai condiments, tested on bacteria by disk diffusion assay

Thai condiments	<i>V. parahaemolyticus</i>	<i>E. coli</i> 29522	<i>S. aureus</i> 29523
1. Galangal	13.6 ± 0.5 ^a	0	0
2. Lemon grass	0 ^b	0	0
3. Kuffer leaf	0	0	0
4. Garlic	11.6 ± 0.5	20.0 ± 0.7	27.3 ± 0.5
5. Red onion	0	0	0
6. Red chili	0	0	0
7. Green chili	0	0	0
8. Ginger	0	0	0
9. Lemon	8.6 ± 1.2	6.7 ± 0.5	8.6 ± 0.5
10. Turmeric	0	0	0
11. Peppermint	0	0	0
12. Onion	0	0	0
13. Krachai	0	0	0
14. Tetracycline	23.0 ± 0.2	23.0 ± 0	33.0 ± 0

1.5×10^8 cfu/ml of bacteria was spread on MHA, 10 µl of freshly squeezed extract/disk was investigated. Plates were incubated at 37°C for 24 h.

^aThe values represent mean ± SD (mm) of diameter of clear zone including diameter of disk (6 mm) from three experiments.

^bNo clear zone produced.

collected and sterilized by filtration through a 0.45-µm millipore filter.

2.3. Extraction and purification

Galangal was extracted by a method that has been described previously (Tewtrakul et al., 2003). Briefly, galangal was extracted with chloroform, methanol or hot water. Solvents were removed under reduced pressure. An essential oil extract was obtained by hydrodistillation. The pure compound, 1'-acetoxychavicol acetate, was isolated by chromatographic techniques and the spectroscopic data compared with previously described results (Itokawa et al., 1981). The extracts and pure substance were dissolved in dimethyl sulphoxide (DMSO) before assay.

2.4. Screening for inhibitory activity of Thai condiments on *V. parahaemolyticus*

Using the disk diffusion technique (National Committee for Clinical Laboratory Standards (NCCLS), 1995), 10 µl of each freshly squeezed extract of 13 Thai condiments was impregnated on 6.0-mm diameter sterilized disks (Macherrey-Nagel, Germany). These disks were placed on the surface of Muller Hinton agar (MHA) + 1% NaCl (20 ml per plate) which were already inoculated with a pandemic strain of *V. parahaemolyticus* PSU 335. In some experiments, PSU 414, PSU 253 and PSU 1702 were added. *E. coli* ATCC 29523 and *S. aureus* ATCC 29522 were used as standard controls. The plates were incubated at 37°C and examined after 24 h for a zone of inhibition. The diameter of the inhibition zone was recorded. Tetracycline was used in assays as a standard control drug.

2.5. Inhibitory activity of extracts and pure substance of galangal on *V. parahaemolyticus*

DMSO solutions containing 25, 50 and 100 µg of the chloroform-, methanol- and water-extracted compounds from galangal were each impregnated on a sterilized disk. Essential oils and pure substances at concentrations of 100–1000 µg/disk were also tested. The inhibitory activity on the pandemic strain of *V. parahaemolyticus* PSU 335 was evaluated by the disk diffusion technique as previously described.

2.6. Stability of galangal

In order to investigate the inhibitory activity of galangal and whether it will remain after processing, freshly squeezed extracts of galangal were subjected to freeze or spray drying. Briefly, in the freeze-dried process, the extract was frozen and dried in a freeze-dry system (Labconco, USA). In the spray-dried process, the extract was mixed with maltodextran and was sprayed in the spray dryer (Niro, Denmark) at 110 °C, 2 rpm for 2 h. They were reconstituted to the original concentrations and inhibitory activities were evaluated by the disk diffusion and agar-well diffusion methods.

2.7. Physicochemical treatment of galangal

To investigate the efficacy of galangal in gastric acid and bile salts, either PBS pH 3 or 0.3% bile salt was added into the fresh preparation of galangal at a ratio of 1:1. They were kept at 37 °C for 30 min and their inhibitory activity against *V. parahaemolyticus* PSU 335 was evaluated by the agar-well diffusion technique (Reddish, 1929). Briefly, 1.5×10^8 cfu/ml of *V. parahaemolyticus* was spread on MHA + 1% NaCl. Wells were punched in the agar and each was filled with 50 µl of treated extract. The plates were incubated at 37 °C and examined after 24 h. The diameter of the inhibition zone was recorded. To determine the effect of temperature, fresh preparations of galangal were heated in a water bath at either 50 or 100 °C for 30 min, and the inhibitory activity was evaluated by the agar-well diffusion technique.

2.8. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of galangal

The tube dilution method was employed to determine the lowest concentration of freshly squeezed extract of galangal that can inhibit growth of bacteria (MIC) and the lowest concentration that can kill the bacteria (MBC) (Finegold et al., 1978; McGinnis and Rinaldi, 1991). The extract was diluted twofold in sterile MHB + 1% NaCl in a test tube and inoculated with 0.5 ml containing 2.5×10^5 cfu of *V. parahaemolyticus* PSU 335. Tubes were incubated at 37 °C overnight and the highest dilution, where no growth was evident, was recorded as the MIC.

For MBC testing, a loopful of broth was taken from the tube containing no growth and inoculated onto MHA + 1% NaCl and incubated overnight at 37 °C. The highest dilution where there were no survivors was recorded as the MBC. In both of the above methods, a solvent control, DMSO, was included.

2.9. Statistical analysis

In each test, three experiments were carried out and each experiment was done in duplicate. Student's *t* test was used for comparison of the results.

3. Results

Thirteen popular Thai condiments were screened for inhibitory activity against the pandemic strain of *V. parahaemolyticus* PSU 335. Freshly squeezed extracts of three condiments, galangal, garlic and lemon showed inhibitory activity with a diameter of the clear zone of 13.6 ± 0.5 , 11.6 ± 0.5 and 8.6 ± 1.2 mm, respectively (Table 1). Suppression of growth of *V. parahaemolyticus* by galangal, garlic and lemon was confirmed using the pandemic strain of *V. parahaemolyticus* PSU 414 and non-pandemic strains PSU 253 and PSU 1702. It was found that garlic was the most effective herb that inhibited *V. parahaemolyticus* ($P < 0.001$), and galangal was superior to lemon ($P < 0.001$) (Table 2). Although the two non-pandemic strains (PSU 253 and PSU 1702) showed less inhibitory effect by *garlic and lemon, respectively, the average mean of these two strains was not significantly different from those of the pandemic strains (PSU 335 and PSU 414) ($P > 0.05$).

Most of the popular Thai spicy soups use seafood for cooking, and contain galangal. Therefore, this herb was extracted by chloroform, methanol and water and the

Table 2
Inhibitory activities of freshly squeezed extract of galangal, lemon and garlic on pandemic and non-pandemic strains of *V. parahaemolyticus*

Bacteria strain	Virulence factors		Galangal ^a	Lemon ^b	Garlic ^c
	<i>tdh</i>	<i>trh</i>			
<i>V. parahaemolyticus</i> PSU 335	+ve	-ve	11.3 ± 0.9^d	9.0 ± 0.8	16.6 ± 1.2
<i>V. parahaemolyticus</i> PSU 414	+ve	-ve	12.3 ± 0.5	9.3 ± 0.5	17.0 ± 2.2
<i>V. parahaemolyticus</i> PSU 253	-ve	-ve	13.5 ± 0.5	9.0 ± 1.4	11.0 ± 0.8
<i>V. parahaemolyticus</i> PSU 1702	+ve	+ve	13.3 ± 0.5	8.0 ± 0	17.7 ± 0.5
Tetracycline			22.0 ± 0.5		

^a10 µl of each was investigated.

^bSignificantly different from galangal and garlic ($P < 0.001$).

^cSignificantly different from galangal and lemon ($P < 0.001$).

^dMean \pm SD (mm) of diameter of clear zone.

Table 3

The inhibitory effect of chloroform, methanol and water extracts of galangal on pandemic strains of *V. parahaemolyticus* by disk diffusion technique

Concentration/disk	Chloroform	Methanol	Water
25 µg	9.5±0.5 ^a	0 ^b	0
50 µg	12.0±0	0	0
100 µg	13.5±0.5	0	0
Freshly extract		11.5±0.2	
Tetracycline		22.5±0.5	

1.5 × 10⁸ cfu/ml of bacteria was spread on MHA, extracts at concentration of 25, 50 and 100 µg/disk or 10 ml/disk for freshly squeezed extract were placed on the surface of the agar and the plates were incubated at 37 °C for 24 h.

^aMean ± SD (mm) of diameter of clear zone.

^bNo clear zone detected.

Table 4

The effect of l'-acetoxychavicol acetate and essential oil extracted from galangal on *V. parahaemolyticus* by the disk diffusion technique

Concentration (µg/disk)	l'-acetoxychavicol acetate	Oil
1000	11.7±0.2 ^a	0
500	9.2±0.2	0
400	0	0
300	0	0
200	0	0
100	0	0
Freshly extracted ^b	12.2±0.5	
Tetracycline	22.7±0.2	

^aMean ± SD (mm) of diameter of clear zone.

^b10 µl of freshly squeezed extract/disk was investigated.

inhibitory activity of galangal extracts on the pandemic strain *V. parahaemolyticus* PSU 335 was determined. Using the disk diffusion technique, the galangal chloroform extract displayed zones of inhibition of 9.5±0.5, 12.0±0 and 13.5±0.5 mm at concentrations of 25, 50 and 100 µg/disk, respectively (Table 3). To evaluate whether the inhibitory effect was due to essential oils, the oil obtained by hydrodistillation of galangal was investigated. No inhibitory activity on *V. parahaemolyticus* was observed (Table 4). However, the substance obtained after further purification of the chloroform extract by chromatography showed an inhibitory effect on the pandemic strain of *V. parahaemolyticus* at concentrations of 500 and 1000 µg/disk which are higher than those compared to the fresh preparation.

This prompted us to evaluate the possibility of using freshly prepared galangal in food. A variety of methods to prepare galangal in food production were examined to evaluate the stability of the inhibitory activity of galangal on *V. parahaemolyticus*. Using the agar-well diffusion technique, we found the diameter of the zone of inhibition of a freshly squeezed preparation was significantly reduced

Table 5

Inhibitory activity of galangal after processing

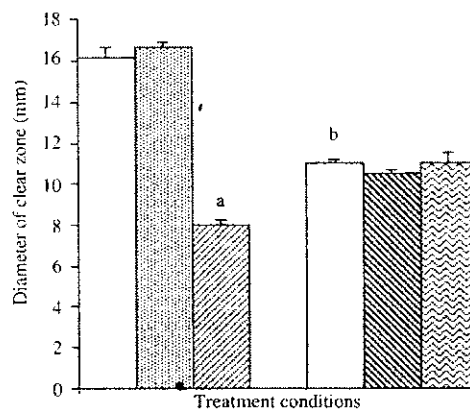
	Disk technique			Agar-well ^a technique
	10 µl	20 µl	30 µl	
Freshly extracted	9.0±0 ^b	9.2±0.5	11.2±0.2	16.0±0 ^c
Freeze dried ^d	0	6.7±0.2	7.7±0.2	13.5±0
Spray dried ^d	0	0	0	8.5±0.5
Tetracycline	20.5±0			

^a50 µl/well.

^bMean ± SD (mm) of diameter of clear zone.

^cSignificantly different from freeze dried and spray dried ($P < 0.005$ and $P < 0.001$, respectively).

^dAqueous freshly squeezed extract was subjected to freeze drying and spray drying.



□ Control ■ Heated 50°C ▨ Heated 100°C □ Control ▨ pH 3 ▨ Bile 0.15%

Fig. 1. The effect of heat treatment, acidic condition and bile salt concentration on inhibitory activity of freshly squeezed galangal on a pandemic strain of *V. parahaemolyticus* by the agar-well diffusion technique: (a) significantly different from control ($P < 0.001$) and (b) control was added with 1% NaCl at a ratio of 1:1.

from 16.0±0 to 13.5±0 mm (15.6% reduction) and 8.5±0.5 mm (46.9% reduction) by freeze and spray drying, respectively. This was confirmed in the disk diffusion technique at a concentration of 10–30 µl/disk (Table 5). This indicates that when water was removed in the freeze-drying process, some of the active compounds were degraded and the high temperature during spray drying also destroyed the activity. Heating the freshly squeezed extract to 100 °C for 30 min significantly reduced the inhibition level of *V. parahaemolyticus* ($P < 0.005$) whereas no reduction in inhibitory activity was observed at 50 °C (Fig. 1). Treatment of freshly squeezed galangal extract at pH 3 or administration of bile salt at a final concentration of 0.15% which are the conditions of gut after food consumption had no effect on the suppression of growth of *V. parahaemolyticus*. The antibacterial activity of freshly squeezed extract of galangal was confirmed by MIC and MBC which were 1:16 and 1:16, respectively.

4. Discussion

Plants with antimicrobial activities have become more interesting because some of them are part of the arsenal of modern antimicrobial drugs and many people are aware of problems associated with the over-prescription and misuse of traditional antibiotics. In this study, the antimicrobial activity of herbs used as Thai condiment was investigated. We found only three ingredients: galangal, garlic and lemon suppressed growth of *V. parahaemolyticus*. Their inhibitory activities were confirmed and suppression was not specific to the pandemic strain because the growth of both pandemic and non-pandemic strains of *V. parahaemolyticus* was suppressed. Interestingly, in this study galangal had no effect on *E. coli* and *S. aureus*. Oonmetta-aree et al. (2006) found no antibacterial activity of galangal on *E. coli* but for *S. aureus* its ethanol extract exhibited MIC and MBC at a concentration of 0.325 and 1.3 mg/ml, respectively. Therefore, as we used only 10 µl of galangal aqueous extract /disk, we might not expect to detect any activity with *S. aureus*.

As galangal is frequently used in Thai spicy soup (Tom yum), this led us to further investigations. Only a chloroform extract of galangal showed inhibitory activity in a dose-response manner (Table 3). Essential oil components of some spices are known to exhibit antibacterial properties (Dean and Richie, 1987; Aureli et al., 1992; Hammer et al., 1999; Wannissorn et al., 2005). Essential oil extracted from galangal was evaluated, but no suppression of *V. parahaemolyticus* growth was detected at the concentration of 1000 µg/ml (Table 4). However, in this study one purified component derived from the chloroform extract exhibited inhibitory activity on *V. parahaemolyticus* at concentrations of 500 and 1000 µg/disk. It was identified as 1'-acetoxychavicol acetate, and this has also been reported to possess antimycobacterium activity with MIC of 0.024 µg/ml (Phongpaichit et al., 2006). However, in this study, the inhibitory concentration of *V. parahaemolyticus* was very high, so although 1'-acetoxychavicol acetate was the main product obtained after purification, other compounds in the fresh extract and chloroform extract may be responsible for the inhibition of *V. parahaemolyticus*. Therefore, these active components may have been lost during extraction and purification. This was confirmed as a lower inhibitory activity was detected with a chloroform extract compared to a freshly squeezed extract (Table 3). Although 1'-acetoxychavicol acetate is one component identified in essential oil (De Pooter et al., 1985), in this study we were unable to detect any effect of essential oil on *V. parahaemolyticus* at the concentrations used.

Galangal is not only widely used in Thailand but in India, China and Southeast Asian countries, such as Indonesia and Philippines. Apart from its use as a spice, it is also accepted as a traditional medicine for several purposes, such as treating problems associated with the digestive system, for relieving bronchitis, flatulence, fungal infections and itching (Grieve, 1989; Xia, 1989; Brown,

1995). In this study, the MIC of freshly squeezed extract was 1:16. As the inhibitory activity of galangal was reduced after boiling at 100°C for 30 min (Fig. 1), and the inhibitory activity of fresh preparation was superior to the chloroform extract, we suggest that it is better to use a fresh preparation in cooking to maintain the active component which is not destroyed by an acidic condition and bile salt concentration. Inhibitory properties of galangal against pandemic strains of *V. parahaemolyticus* may promote countries that face problems from this organism to use this herb or evaluate their local herb to control infection.

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