Bioautography-guided isolation of antibacterial compounds of essential oils from Thai spices against histamine-producing bacteria

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Abstract: The outbreak of histamine fish poisoning has been being an issue in food safety and international trade. The growth of contaminated bacterial species including *Morganella morganii* which produce histidine decarboxylase causes histamine formation in fish during storage. Histamine, the main toxin, causes mild to severe allergic reaction. At present, there is no well-established solution for histamine fish poisoning. This study was performed to determine the antibacterial activity of essential oils from Thai spices against histamine-producing bacteria. Among the essential oils tested, clove, lemongrass and sweet basil oils were found to possess the antibacterial activity. Clove oil showed the strongest inhibitory activity against *Morganella morganii*, followed by lemongrass and sweet basil oils. The results indicated that clove, lemongrass and sweet basil oils could be useful for the control of histamine-producing bacteria. The attempt to identify the active components using preparative TLC and GC/MS found eugenol, citral and methyl chavicol as the active components of clove, lemongrass and sweet basil oils, respectively. The information from this study would be useful in the research and development for the control of histamine-producing bacteria in fish or seafood products to reduce the incidence of histamine fish poisoning.

Keywords: TLC bioautography, antibacterial activity, histamine-producing bacteria, essential oils, Thai spices.

INTRODUCTION

Histamine fish poisoning is a foodborne chemical intoxication caused by bacterially contaminated fish or seafood. Scombroid fish (Scombridae) including tuna, mackerel and bonito, which contain high amounts of free histidine in their muscle have been implicated in histamine fish poisoning. Other fish species, such as mahi-mahi, sardines, herring, pilchards, anchovies, bluefish, salmon and swordfish are also related to histamine fish poisoning outbreaks (Phuvasate and Su, 2010; Hungerford, 2010). The growth of bacterial species including *Morganella morganii*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* which produce histidine decarboxylase causes the formation of histamine in fish during storage. This main toxin, histamine, causes mild to severe allergic reactions. Histamine is heat-resistant and stable, thus it could not be eliminated by freezing, cooking or smoking processes. The rate of histamine production in fish increases when exposed to higher temperatures. At present, the best practice to prevent histamine fish poisoning is soaking fish in ice or cold seawater immediately after catching and keeping fish at 4°C. However, histamine-producing bacteria can still grow slowly under low temperature (Phuvasate and Su, 2010; Sangcharoen *et al.*, 2009). The disease is an important issue for food safety, public health concern and international trade. This incidence has stimulated food producers, distributors, restaurants and all food handlers to be more cautious about the safety of food products. Due to the lack of the proper method of preservation, handling and storage process, histamine fish poisoning is still being a world outbreak (Lehane and Olley, 2000). At present, there is no well-established solution for histamine fish poisoning. Thus, the search for suitable prevention is urgently needed to decrease its incidence.

Thai spices, mainly consisting of essential oils, have been involving in Thai cuisine for centuries, especially when cooking seafood dishes. Essential oils from Thai spices have been proved to exert interesting biological activities including antibacterial and antioxidant activities (Prasad *et al.*, 1986; Onawunmi *et al.*, 1984; Wannissorn *et al.*, 2005; Sartoratto *et al.*, 2004; Hussain *et al.*, 2008; Hussein Ayoub, 1990). There are only few studies on this activity (Sangcharoen *et al.*, 2009; Husain, 1996; Wendakoon and Sakaguchi, 1992). However, no extensive study on the compounds responsible for the antibacterial activity against *M. morganii* was reported. Previous studies indicated that the studies on Thai spices were not completed. Therefore in this study, the essential oils of Thai spices will be extensively studied to determine the activity of the oils and their active components**.**

MATERIALS AND METHODS

Materials

The essential oils used in this study were obtained from galanga rhizomes (*Alpinia galanga* (L.) Willd.), fingerroot rhizomes (*Boesenbergia pandurata* (Roxb.)

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Schltr.), kaffir lime leaves and peels (*Citrus hystrix* DC.), lemongrass stems (*Cymbopogon citratus* Stapf), sweet basil leaves (*Ocimum basilicum* L.), holy basil leaves (*Ocimum tenuiflorum* L.), betel leaf vine (*Piper betle* L.) and clove buds (*Syzygium aromaticum* (L.) Merr. & LM Perry). All essential oils were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd. (Bangkok, Thailand). The major compounds of essential oils; trans-caryophyllene, cineole, citral, citronellol, citronellal, eugenol, geraniol, linalool, methyl chavicol and β-myrcene were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Methyl cinnamate was obtained from Tokyo Chemical Industry (Tokyo, Japan).

Bacterial cultures

Morganella morganii was obtained from Assistant Professor Pongtep Wilaipun, Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Thailand. The bacterium was grown on tryptic soy agar (TSA) medium at 25°C.

Broth dilution assay

The ability of the plant essential oils to inhibit bacterial growth in broth was determined using the standard procedure to evaluate the minimum inhibitory concentration (MIC) according to the method of Percival, *et al*. (2006) with some modifications. Antibacterial activity was determined by microdilution assay on 96-well polystyrene microtiter plate. All essential oils were dissolved in 95% ethanol and tween 80, and then two-fold serial dilution was performed in 96-well microtiter plate. The optical density (600 nm) of prepared inoculums was adjusted to 0.2 (approximately 10^7 CFU/mL). An aliquot of test microorganisms was added equally into each well. Plate was incubated under aerobic condition at 25°C for 24 h and the MICs were determined. Then, the bactericidal effect was assessed by the minimum bactericidal concentration (MBC) determination. Samples were removed from wells of MIC microtiter plate that showed no turbidity and were dropped onto TSA plate, then incubated at 25°C for 24 h. The concentration which showed no visible growth was reported as MBC.

GC/MS analysis

The active essential oils were analyzed by gas chromatography-mass spectrometry (GC/MS) analysis on Shimadzu QP2010 apparatus using a DB-5ms (J&W Scientific, Folsom, CA) bonded phase fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The oven temperature programs for clove, lemongrass and sweet basil oils were modified from the method of Della Porta *et al* (1998), Inouye *et al* (2001) and Hussain, *et al*. (2008), respectively. He at a flow rate of 0.68 mL/min was used as carrier gas. The injection port was set at 250°C. Significant quadrupole MS operating parameters: interface temperature 250°C; electron impact ionization at 70 eV with scan mass range of 40-400 *m/z* at a sampling rate of 1.0 scan/s, were used. Compounds were identified by computer search using the Wiley and NIST digital libraries of mass spectral data and by comparison of their retention times and authentic mass spectra with reference standards.

Determination of the active compounds of plant essential oils

The active components of plant essential oils were identified by bioautography using thin layer chromatography (TLC) by modified method of Chomnawang, *et al*. (2005). Three silica gel GF_{254} plates (A, B, C) were prepared. Plate A was used as a reference chromatogram, plate B was used for bioautography method, and plate C was prepared for the isolation of plant active compounds. Three plates were subjected to separation by using toluene – ethyl acetate (93:7 v/v) as developing solvent. After development, plate A was dried in fume hood, sprayed with anisaldehyde sulfuric reagent and heated at 110°C for 5-10 min to detect the isolated components. Rf value of each compound was compared with those of the reference standards.

After the solvent was completely removed, plate B was placed on the top of agar base. The inoculum of tested strain containing approximately 10^7 CFU/mL in molten TSA was distributed over the plate. After incubated at 25°C for 24 h, plate B was detected for the clear zones which were the indication of antibacterial activity. The identification of active components was based on the comparison of Rf of the corresponding spot with Rf of reference standard on plate A. Plate C was used as preparative TLC.

To isolate the active components, the inhibition zones were scraped off the plate, then the active component was eluted from the silica gel plate with hexane and the eluate was evaporated to dryness under *vacuo*. Then, the active components were confirmed by GC/MS as mentioned above. The identified active components of essential oils were further evaluated for their antibacterial activity using broth dilution assays.

RESULTS

The MICs and MBCs of essential oils were evaluated as shown in table 1. The results revealed that clove oil exhibited the strongest antibacterial activity against *M. morganii* with MIC of 0.13% v/v and MBC of 0.25% v/v. Sweet basil and lemongrass oils also showed the inhibitory activity against the test strain.

Clove, lemongrass and sweet basil oils which exhibited strong antibacterial activity were analyzed by GC/MS. The chemical constituents of active essential oils were identified as shown in tables 2-4. The results indicated that the major compounds of clove, lemongrass and sweet

Table 1: MICs and MBCs of 9 essential oils and their active compounds against *M. morganii*

M. morganii		Galanga	Fingerroot	Kaffir lime leaf	Kaffir lime peel	Lemongrass	Sweet basil	Holy basil	Betel leaf vine	Clove	Eugenol	Citral	Methyl chavicol
MIC	µL/mL	>5.00	>5.00	>5.00	>5.00	5.00	2.50	>5.00	>5.00	1.25	1.25	1.25	5.00
	% v/v	>0.50	>0.50	>0.50	>0.50	0.50	0.25	>0.50	>0.50	0.13	0.13	0.13	0.50
	mg/mL	>4.60	>4.46	>4.25	>4.35	4.40	2.39	>4.88	>5.17	1.31	1.33	1.11	4.83
MBC	µL/mL	>5.00	>5.00	>5.00	>5.00	5.00	5.00	>5.00	>5.00	2.50	2.50	2.50	>5.00
	% v/v	>0.50	>0.50	>0.50	>0.50	0.50	0.50	>0.50	>0.50	0.25	0.25	0.25	>0.50
	mg/mL	>4.60	>4.46	>4.25	>4.35	4.40	4.77	>4.88	>5.17	2.63	2.67	2.22	>4.83

basil oils were eugenol, citral (*Z*-citral and *E*-citral) and methyl chavicol, respectively.

Table 2: Chemical constituents of clove oil by GC/MS analysis

Peak #	Retention Time (min)	Area %	Name
1	44.714	96.24	Eugenol
2	48.897	1.06	Caryophyllene
3	93.509	2.70	Unidentified

Table 3: Chemical constituents of lemongrass oil by GC/MS analysis

Peak #	Retention Time (min)	Area %	Name
1	10.734	2.13	β-Myrcene
2	20.013	1.40	Limonene oxide
3	22.751	38.13	*Z*-Citral or Neral
4	23.333	3.66	Geraniol
5	24.148	52.88	*E*-Citral or Geranial
6	29.169	1.80	Geranyl acetate

Based on the results of MIC and MBC assay, clove, lemongrass and sweet basil oils were selected for TLC bioautography assay and GC/MS technique to identify the active component responsible for their antibacterial activity. TLC chromatogram showed that the active component of clove oil was eugenol. The identification was confirmed by preparative TLC and GC/MS analysis. The result revealed that the active fraction of clove oil was eugenol. By the same method, the active compounds of lemongrass and sweet basil oils were confirmed by GC/MS analysis as citral (*Z*-citral and *E*-citral) and methyl chavicol, respectively. All the active compounds exhibited antibacterial activity against *M. morganii* as shown in table 1. However, their MICs and MBCs were higher than 1.31 mg/mL which was over the cut-off limit for pure compounds (10-12.5 µg/mL) recommended by the United Nations Industrial Development Organization (UNIDO) (Dhawan and Srimal, 1998).

Table 4: Chemical constituents of sweet basil oil by GC/MS analysis

Peak #	Retention Time (min)	Area %	Name
1	9.514	0.20	β-Myrcene
2	10.982	0.06	Limonene
3	11.100	1.06	1,8-Cineole
4	11.601	1.81	trans-β-Ocimene
5	13.889	0.16	Linalool
6	15.879	0.71	Camphor
7	17.804	90.12	Methyl chavicol
8	26.982	0.69	β-Elemene
9	27.385	0.42	Methyl eugenol
10	28.741	2.92	α-Bergamotene
11	28.808	0.15	α-Guaiene
12	29.622	0.13	α-Humulene
13	30.709	0.25	Germacrene D
14	31.658	0.28	δ-Guaiene
15	32.196	0.42	γ-Cadinene
16	40.019	0.62	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene

DISCUSSION

The outbreak of histamine fish poisoning leads to several studies on antibacterial agents against histamine-producing bacteria. In this study, clove, lemongrass and sweet basil oils were found to possess antibacterial effect against histamine-producing bacteria. The antibacterial effect of lemongrass oil supported the fact previously observed by Sangcharoen, *et al*. (2009). The MIC and MBC assay used in this study is more suitable than disk diffusion assay. As the essential oils are water insoluble, the observed size of inhibition zones in disk diffusion assay which is aqueous base was smaller than actual result. The strongly active essential oils were selected for biological guided separation to identify the active compounds. GC/MS analysis revealed that the compounds responsible for antibacterial activity against histamine-producing bacteria of clove, sweet basil and

lemongrass oils were eugenol, methyl chavicol and citral (*Z*-citral and *E*-citral), respectively. All of the active compounds were found to be major constituents of the essential oils. Our result on clove oil supported previous report that clove inhibited bacterial growth with eugenol as active component. The potency of the oil and eugenol were almost the same owing to the high content (96.24%) of eugenol in the oil. Eugenol was reported to be able to inhibit several bacterial growth e.g. *Escherichia coli, Bacillus cereus, Salmonella* spp., *Listeria monocytogenes, E. aerogenes* and *M. morganii* (Tajkarimi *et al.,* 2010; Burt, 2004; Dorman and Deans, 2000; Wendakoon and Sakaguchi, 1992). The phenolic group in eugenol is attributed to its activity (Devi *et al.,* 2010; Thoroski *et al.*, 1989). There are four chemical races of sweet basil which are camphor, eugenol, methyl chavicol and methyl cinnamate races. Thai sweet basil was reported to be methyl chavicol race (Chokechaijaroenporn, 1991). The result showed that sweet basil oil contains methyl chavicol (90.12%). The oil exhibited antibacterial activity with MIC 2.50 μL/mL while MIC of methyl chavicol was 5.00 μL/mL. This result indicated that there were some other active compounds in the oil and suggested that the oil was more potential for commercial product than methyl chavicol. Basil methyl chavicol has been reported to be active against *Aeromonas hydrophila* and *Pseudomonas fluorescens*, natural flora of fresh lettuce (Burt, 2004). This is the first report on the activity against histamine-producing bacteria. The result of lemongrass oil from our study found that the oil which contains *E*-citral (52.88%) and *Z*-citral (38.13%) exhibited weaker activity than citral. The result suggested that other constituents in lemongrass oil could exert antagonistic effect. Previous reports found that citral demonstrated the antibacterial activity against several pathogens, such as *Salmonella typhimurium, B. cereus, E. coli, L. monocytogenes, Salmonella* spp. and *Vibrio vulnificus* (Tajkarimi *et al.,* 2010; Burt, 2004; Dorman and Deans, 2000; Kim *et al.,* 1995). This study is the first report on the active compound responsible for the antibacterial activity against histamine-producing bacteria of lemongrass oil. Further evaluation on the antibacterial activity of all of the active compounds by MIC and MBC assay were performed on reference compounds due to the instability of isolated compounds. The MICs and MBCs of all active compounds were higher than the cut-off limit for pure compounds recommended by UNIDO. Thus, pure active components showed less potential for product development. In conclusion, the antibacterial activity against histamine-producing bacteria of essential oils from Thai spices has been reported for the first time except for lemongrass oil. Moreover, this study is the first report on the active components of Thai essential oils against histamine-producing bacteria. The result of the present study and previous reports suggested that clove, lemongrass and sweet basil oils are the promising antibacterial oils against histamine-producing bacteria. The information from this study would be useful in the research and development for the control of histamine-producing bacteria in fish or seafood products to reduce the incidence of histamine fish poisoning.

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