

ANTIMICROBIAL ACTIVITY OF SOME THAI AROMATIC PLANTS AGAINST ORAL PATHOGENS INDUCING HALITOSIS

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ABSTRACT

Objective: The objective of the present work is to analyze that the extracts of 25 Thai aromatic plants used in Thai food recipes were subjected to evaluated antimicrobial activity against *Fusobacterium nucleatum* and *Streptococcus mutans* which have been known as bacterial inducing halitosis.

Methods: Disk diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) by broth microdilution were used to determine the antibacterial activity.

Results: The results revealed that three plant extracts, namely *Piper betle* Linn., *Eupatorium stoechadosmum* Hance, and *Alpinia galangal* (L.) Wild can inhibit the growth of both halitosis inducing bacteria with strong activity. Among these, the extract of *P. betle* Linn. is the most effective in inhibiting both bacteria followed by *E. stoechadosmum* Hance and *A. galangal* (L.) Wild, respectively. The MIC (mg/ml) values of the extracts which can inhibit *F. nucleatum* were ranging from 1.56 to 12.50, and the MBCs (mg/ml) values were ranging from 3.125 to 25.00. In addition, the MICs and MBCs of the extracts which can inhibit *S. mutans* were found that 1.56–25.00 and 3.125–50.00, respectively.

Conclusion: The extracts obtained from *P. betle*, *A. galangal*, and *E. stoechadosmum* displayed good activity against *F. nucleatum* and *S. mutans* and these extracts could be a potential source of new antimicrobial agents.

Keywords: Antimicrobial activity, Thai aromatic plants, *Fusobacterium nucleatum*, *Streptococcus mutans*, Halitosis.

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INTRODUCTION

Halitosis, medically name of bad breath, or oral malodor, exhaled from the oral cavity. Halitosis is a significant social and psychological problem [9]. The intrinsic causes of halitosis are both oral cavity (90%) and systemic disorder (10%) [17]. Halitosis as the results of inadequate plaque control, tongue coating, periodontal disease, dental caries, oral candidiasis, oral infections, accumulation of food debris, and dry mouth [6]. The major compounds that cause halitosis are volatile sulfur compounds (VSCs) such as hydrogen sulfide and methyl mercaptan, which are produce from microbial metabolism of sulfur-containing amino acid. *Fusobacterium nucleatum*, Gram-negative anaerobic oral bacteria, has pathogenic potential and is implicated in periodontal diseases. It produces large amounts of VSCs and is a representative for the development of halitosis. On the other hand, bad breath has been reported to be caused by dental caries [13]. Dental caries causes acid formation by cariogenic bacteria such as *S. mutans* and results from the interaction of *S. mutans* and other related bacteria by production of biofilm on tooth surfaces [12].

Halitosis can be treated by mechanical and chemical methods. In addition, many products such as mouthwash, dentifrice, gum, tablets, and lozenges can play supporting roles in controlling oral malodor. Many methods can reduce halitosis by decrease microbial accumulation, nutrient availability, exerting anti-inflammatory, and masking the bad breath. The active ingredients used for controlling halitosis can be divided into chemical agents and naturally derived compounds. However, most chemical compounds have been known to induce side effects when prolong used. For example, chlorhexidine (CHX) stains the teeth and induces brownish discoloration of the pellicle covering teeth and tongue, alterations in test, increased desquamation of oral mucosa, and increase calculus formation [8]. To reduce the side effects of the chemical agents, the role of natural products may provide an alternative

approach to develop new treatments. Aromatic plants are also known as herbs and spices and have been used since ancient times as folk medicine and as a preservative in foods. They contain many biologically active compounds which have been found to possess antimicrobial, antioxidant, and anti-inflammatory properties.

Nowadays, the demand for these plants has increased because they are natural, eco-friendly and generally believed to be safer than synthetic agents. Therefore, antimicrobial efficacies of aromatic plants synergistic with their aromatic effects are appreciable for halitosis neutralization and flavoring the oral care products as well [15]. Thailand is located in the tropical region, resulting in the variety of plants, including numerous species of aromatic plants. These plants are rich in various kinds of aromatic compounds. It is, therefore, interesting to investigate the effect of aroma compounds to inhibit microorganisms inducing halitosis. In this research is to evaluate the antimicrobial activity of some Thai aromatic plants traditionally used in Thai food recipes against *F. nucleatum* and *S. mutans*.

MATERIALS AND METHODS

Plant materials

Twenty-five Thai aromatic plants were purchased from a local market in Chiang Mai, Thailand. They were identified botanically at the Biology Department of Chiang Mai University, Chiang Mai, Thailand.

Preparation of plant extract

All of the fresh plants in this were cleaned and cut into small pieces before drying at 50°C in a hot air oven. At the end of drying, the final moisture content of dried plants determined by gravimetrically method was not exceeded 10% (dry basis). Dried plants were ground to get a fine powder using a mechanical blender and kept separately in plastic bags at room temperature before extraction. Dry plants were macerated

with 80% ethanol [11] in the dark for 3 days at room temperature. At the end of extraction, each mixture was filtered using filter paper Whatman No.1 (Whatman International Ltd., Middlesex, England) and then was evaporated to dryness under reduced pressure in a vacuum rotary evaporator (Buchi Rotavapor R-114, Switzerland). The dried plant extracts were stored at 4°C in airtight sterile bottle, protected from light until used. The stock of plant extracts was dissolved in dimethyl sulfoxide (DMSO) to get 400 mg/ml concentration of each extract and used as a sample for determination of antimicrobial activity.

Microorganisms and culture media

The organisms used in this study were as followed: *F. nucleatum* (JMC 8532 or ATCC 10953) and *S. mutans* (ATCC 25175). These were kindly provided by the Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Japan and the laboratory of Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand, respectively. All media were purchased from Merck (Merck, Germany). Hemin and Vitamin K was purchased from Remel (Remel, Lenexa, KS).

Antimicrobial assay

Disk diffusion method

Antimicrobial activity was determined by agar disk diffusion method [5]. *Fusobacterium nucleatum* was cultured in brain heart infusion (BHI) broth supplemented with 1.0% (v/v) hemin and 0.1% (v/v) Vitamin K for 72 h at 37°C under anaerobic condition (10% H₂, 10% CO₂, and 80% N₂). The inoculum suspension was prepared which adjusted their turbidity to 1.0×10⁸ CFU/ml equivalent to McFarland standard No. 0.5 [19]. Each of inoculum suspension was prepared freshly before use, spread on the surface of agar using a sterile cotton swab moistened with inoculum suspension. Blood agar plate containing hemin and Vitamin K was used for *F. nucleatum*. A Ø 6 mm diameter sterile paper discs (Whatman International Ltd., Middlesex, England) were loaded with 20 µl (400 mg/ml) of plant extract, allowed to dry and placed on the agar surface. Plates were then incubated at 37°C for 72 h under anaerobic condition.

S. mutans was grown in BHI broth at 37°C for 24 h under anaerobic condition and adjusted their turbidity to 1.0×10⁸ CFU/ml. The processes were the same as mentioned above except instead of using BHI plate were incubated at 37°C for 24 h under anaerobic condition. A 0.12% CHX and DMSO were used as negative controls and positive control, respectively.

After incubation, the antimicrobial activity of plant extract was determined by measuring the diameter of the inhibition zone in millimeter. All experiments were done in triplicates.

Minimum inhibition concentration (MIC)

After completion of MIC determination, the MIC results of this experiment were confirmed by minimum bactericidal concentration (MBC) determination. The MBC can be determined by subculturing [4]. One loop full of a sample from each well was transferred on an agar plate and further incubation at 37°C under anaerobic condition for 72 h and 24 h for *F. nucleatum* and *S. mutans*, respectively. The MBC value was recorded as the lowest concentration of plant extract that killed bacteria after incubation.

Statistical analysis

Data were expressed as mean and standard deviation by computational analysis from three independent experiments.

RESULTS

At present, the demand for natural substances is increasing because it is more secure than synthetic materials. Therefore, the search for plants that inhibit bacteria causing bad breath is considered to be interesting research. In the present study, 25 Thai aromatic edible plant extracts were evaluated for their antimicrobial activity against halitosis inducing bacteria (*F. nucleatum* and *S. mutans*). The antimicrobial activity of the extracts and their potency was accessed by the presence and absence of the inhibition zone and zone diameter, respectively, as given in Table 1. Only the alcoholic extract evaluated, as alcohol was found to be a better

Table 1: Antimicrobial activity of Thai aromatic plant extracts against *F. nucleatum*, *S. mutans* using agar disk diffusion method

Plants					Zone of inhibition (mm)	
	Scientific name	Local name	Family	Part used	<i>F. nucleatum</i>	<i>S. mutans</i>
	<i>Coriandrum sativum</i> L.	Coriander (Puk-Chee)	Apiaceae	Leaf	-	8.00±0.70
	<i>Coriandrum sativum</i> L.	Coriander (Puk-Chee)	Apiaceae	seed	-	11.00±0.80
	<i>Centella asiatica</i> (L.) Urban.	Asiatic (Bua-Bok)	Apiaceae	Leaf	-	8.00±0.80
	<i>Eryngium foetidum</i> L.	Long Coriander (Phak chi farang)	Apiaceae	Leaf	-	9.00±0.70
	<i>Anethum graveolens</i> L.	Dill (Puk-Chee-Lao)	Apiaceae	Whole plant	-	14.50±0.40
	<i>Apium graveolens</i> L.	Celery (Khun-Chai)	Apiaceae	Whole plant	-	-
	<i>Allium cepa</i> var. <i>aggregatum</i>	Shallot (Ton-hom)	Alliaceae	Whole plant	-	8.83±0.50
	<i>Eupatorium stoechadosmum</i> Hance	San phra hom (Thai)	Asteraceae	Leaf	15.00±0.60	13.00±0.80
	<i>Cymbopogon citrates</i> (DC.) stapf.	Lemongrass (Tra-Kai)	Gramineae	Rhizome	-	8.11±0.30
	<i>Illicium verum</i> Hook.	Star anise (Poy-kuk)	Illiciaceae	Seed	9.00±1.00	-
	<i>Ocimum gratissimum</i>	Tree basil (Yee-Rha)	Lamiaceae	Seed	-	11.00±0.80
	<i>Pandanus amaryllifolius</i> Roxb.	Pandanus (Toey-hom)	Pandanaceae	Leaf	-	-
	<i>Piper betle</i> L.	Betel Vine (Phlu)	Piperaceae	Leaf	27.00±0.60	25.00±0.80
	<i>Piper nigrum</i> L. (white)	White pepper (Prik-Thai)	Piperaceae	Seed	-	-
	<i>Piper nigrum</i> L. (black)	Black pepper (Prik-Thai)	Piperaceae	Seed	-	-
	<i>Piper retrofractum</i> Vahl.	Long pepper (Dee-Plee)	Piperaceae	Seed	9.00±0.20	-
	<i>Piper sarmentosum</i> Roxb.	Wild betel (Cha-Phlu)	Piperaceae	Leaf	-	-
	<i>Polygonum odoratum</i> Lour.	Vietnamese coriander, (Puk-Preaw)	Polygonaceae	Leaf	-	-
	<i>Citrus hystrix</i> DC.	Leech lime (Ma-Krood)	Rutaceae	Leaf	-	-
	<i>Zanthoxylum limonella</i> Alston	Indian pepper (Ma-khan)	Rutaceae	Seed	-	12.00±0.70
	<i>Alpinia galangal</i> (L.) Wild	Galangal (Kha)	Zingiberaceae	Rhizome	18.00±0.80	23.10±0.90
	<i>Boesenbergia rotunda</i> (L.) Mansf.	Fingerrroot (Kra-Chai)	Zingiberaceae	Rhizome	11.00±0.60	12.00±0.80
	<i>Curcuma longa</i> L.	Turmeric (Kha-min)	Zingiberaceae	Rhizome	-	11.50±0.50
	<i>Zingiber cassumunar</i> Roxb.	Cassumunar ginger (Plai)	Zingiberaceae	Rhizome	-	-
	<i>Zingiber officinale</i> Roscoe	Ginger (King)	Zingiberaceae	Rhizome	10.00±0.60	9.00±0.70
	0.12% chlorhexidine	-	-	-	19.00±0.00	19.90±0.30
	DMSO	-	-	-	-	-

Remarks: Values for the zone of growth inhibition (mm) are presented as mean of triplicates±SD. Interpretation of the plant extracts activity; (-): No inhibition zone, none activity ≤8 mm, 9-11 mm intermediate activity and ≥12 mm strong activity (Bauer et al., 1966) DMSO: Dimethyl sulfoxide, SD: Standard deviation

solvent for extraction of active substances with having antimicrobial activity when compared to water and hexane [1]. In this experiment, the reference standards 0.12% CHX disc showed inhibitory activity against both types of tested bacteria. The DMSO was used as negative control and did not show any activity. The 14 extracts from 25 plants were effective against 1 or 2 tested bacteria. The results showed that seven extracts could effectively inhibit the growth of *F. nucleatum* with the inhibition zone ranging from 27.00 to 9.00 mm. The 12 extracts showed inhibitory activity against *S. mutans* with the inhibition zone ranging from 8.11 to 25.00 mm. In addition, the results also show that there are five extracts that are effective against both types of bacteria. Among these plants, *Piper betle* Linn., *Eupatorium stoechadosmum* Hance, and *Alpinia galangal* (L.) Wild have a strong activity against both types of bacteria. The remaining nine plants had none activity on both types of tested bacteria. Subsequently, the plant extracts that showed inhibitory activity against one or both bacteria was conducted to determine MICs and MBCs. The results are summarized in Table 2. For plant extracts that can inhibit the growth of both bacteria with strong activity revealed that *P. betle* Linn. is most effective in inhibiting both of bacteria followed by *E. stoechadosmum* Hance and *A. galanga* (L.) Wild, respectively. The MIC (mg/ml) values of the extracts which can inhibit *F. nucleatum* were ranging from 1.56 to 6.25, and the MBCs were ranging from 3.125 to 12.50 (mg/ml). In addition, the MICs and MBCs of the extracts which can inhibit *S. mutans* were found that 1.56–25.00 and 3.125–50.00 (mg/ml), respectively. Selective plant extracts will be further carried out for purification and possibility for alternative oral care product.

DISCUSSION

In this study, the antimicrobial activity of 25 Thai aromatic plant extracts against oral pathogens (*F. nucleatum* and *S. mutans*) inducing bad breath. In the study, DMSO and the 0.12% CHX discs were used as negative and positive control, respectively. DMSO without any plant extracts was used as negative control, and none activity could detect for all of the tested bacteria. DMSO consists of a polar part and two nonpolar parts that provide its able to solubilize polar and nonpolar substances and

transpose across hydrophobic barriers. These properties are important for vehicle pharmacological compound that acts in intracellular (Lucas et al., 2017). The 0.12% CHX is an antimicrobial agent. Its antimicrobial action of CHX is due to a positively-charged molecule that binds to the negatively-charged of phospholipids in the inner membrane, leading to increased permeability of the inner membrane and leakage of cell components, such as potassium ions which leading to cell death [10].

The results of this experiment clearly showed that the crude extracts of three plants, namely *P. betle* Linn., *E. stoechadosmum* Hance, and *A. galangal* (L.) Wild revealed a strong inhibitory activity against both of tested bacteria. The results of this experiment are agree with the other reports which related the activity of *P. betle* and *A. galanga*. Among these reports, Deshpande et al. (2013) [20] and Dinesh et al. (2016) [21], who showed that the ethanol extracts of *Piper betel* leaves had inhibitory activities against *S. mutans*. The effects of *P. betle* on *F. nucleatum* were found to be consistent with the report of Ramji et al. (2002) [16] and Babitha et al. (2017) [3]. Similar reports on the antimicrobial activity of *A. galanga* extracts, in this study, were found to be consistent with the report of Teanpaisan et al. (2016) [18] who finding that the ethanolic extract of *A. galangal* has inhibitory activity against both of bacteria. However, the effectiveness of active plant extracts in this study was found to be different from that reported in other studies. This may be due to many factors such as chemical constituents in the extract, vegetative period of the plant (before or after flowering), and environmental factors such as the geo-climatic location and growing conditions, including the concentration of nutrients, temperature, humidity, soil type, and amount of available water [2].

The antibacterial activity of ethanolic leaves extract from *E. stoechadosmum* Hance against *F. nucleatum* and *S. mutans* has not been reported. The bacterial inhibitory properties of this plant may be attributed to the active constituents. Studies on the constituents of this plant have been reported by Dung et al. (1991) [7]. In the report, found that the major constituents of essential oil from *E. stoechadosmum* Hance contained thymohydroquinone dimethyl ether (73.6%), caryophyllene (8.9%) and selina-4,11-diene (11%). Therefore, the study of the chemical constituents of this plant that effects the bacteria are interesting to continue studying.

Table 2: The MIC and MBC of Thai aromatic plant extracts using broth microdilution

Plant extracts	<i>F. nucleatum</i>		<i>S. mutans</i>	
	MIC	MBC	MIC	MBC
<i>Coriandrum sativum</i> L. (leave)	>200	>200	50	100
<i>Coriandrum sativum</i> L. (seed)	>200	>200	50	100
<i>Centella asiatica</i> (L.) Urban	>200	>200	100	200
<i>Eryngium foetidum</i> L.	>200	>200	50	100
<i>Anethum graveolens</i> Linn.	>200	>200	>200	>200
<i>Apium graveolens</i> L.	>200	>200	12.50	25.00
<i>Allium cepa</i> var. <i>aggregatum</i>	>200	>200	50	100
<i>Eupatorium stoechadosmum</i> Hance	6.25	12.50	6.25	12.5
<i>Cymbopogon citratus</i> Stapf.	>200	>200	>200	>200
<i>Illicium verum</i> Hook.	>200	>200	25.00	50.00
<i>Ocimum gratissimum</i>	>200	>200	50	100
<i>Pandanus amaryllifolius</i> Roxb.	>200	>200	>200	>200
<i>Piper betle</i> Linn.	1.56	3.125	1.56	3.125
<i>Piper nigrum</i> L. (white)	>200	>200	>200	>200
<i>Piper nigrum</i> L. (black)	>200	>200	>200	>200
<i>Piper retrofractum</i> Vahl.	>200	>200	>200	>200
<i>Piper sarmentosum</i> Roxb.	>200	>200	>200	>200
<i>Polygonum odoratum</i> Lour.	>200	>200	>200	>200
<i>Citrus hystrix</i> DC	>200	>200	>200	>200
<i>Zanthoxylum limonella</i> Alston	>200	>200	25	50
<i>Alpinia galangal</i> (L.) Wild	6.25	12.50	25.00	50.00
<i>Boesenbergia rotunda</i> (L.) Mansf.	12.50	25.00	50.00	100.00
<i>Curcuma longa</i> L.	>200	>200	50	100
<i>Zingiber cassumunar</i> Roxb.	>200	>200	>200	>200
<i>Zingiber officinale</i> Roscoe	12.50	25.00	25.00	50.00

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

CONCLUSION

In this study demonstrated that extracts obtained from *P. betle*, *A. galanga*, and *E. stoechadosmum* Hance displayed good activity against *F. nucleatum* and *S. mutans*. These plant extracts could be a potential source of new antimicrobial agents. For further study, selective plant extracts will be carried out for purification and possibility useful for alternative oral care product.

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AUTHOR(S) CONTRIBUTIONS

All the authors contributed equally to this manuscript.

CONFLICTS OF INTEREST

Declared none.

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