Antimicrobial Activity of Extracts from a Thai Traditional Remedy Called Kabpi for Oral and Throat Infection and Its Plant Components

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Background: *Thai traditional remedy called Kabpi (KP) has long been used for oral and throat infection. It composed with four herbs Syzygium aromaticum (L.) Merr. et Perry, Nigella sativa L., Limonia acidissima L. and Achasma sphaerocephalum Holtt.*
Objective: *To investigate antimicrobial activity of KP remedy extracts and its plant components.*
Material and Method: *The extract procedures were maceration method with 95% ethanol, 50% ethanol and dried by evaporator, and boiling in water, filtrated dried by lyophilizer. The residue from the maceration in 95% ethanol and 50% ethanol were boiled in water and dried by lyophilizer. In the preliminary studied, all extracts were evaluated antimicrobial activity by disc diffusion method. All extracts were tested against one type of gram positive bacteria Staphylococcus aureus (ATCC 25923) and one type of yeast Candida albicans (ATCC 90028). The active plant extracts were diluted to determine the minimum inhibitory concentration (MIC) values by microtiter plate-based assay.*
Results: *The 95% ethanol extract of KP remedy showed antimicrobial activity against S. aureus (ATCC 25923) and C. albicans (ATCC 90028) (MIC = 0.625, 0.625 mg/ml, respectively). The zone of inhibition of all extracts were in the range of 7.33 to 23.33 mm. The 95% ethanol extract of Achasma sphaerocephalum Holtt. showed the highest inhibition zone against S. aureus 21.00 mm (MIC = 0.625 mg/ml). The 95% ethanol extract of Syzygium aromaticum (L.) Merr. et Perry exhibited the best antimicrobial activity against C. albicans the inhibition zone with inhibition zone of 23.33 mm (MIC = 0.156 mg/ml).*
Conclusion: *The present study demonstrated that the extracts of KP remedy and its plant components had an antimicrobial effect against oral and throat infection such as S. aureus and C. albicans. These results support using this remedy of Thai traditional medicine called Kabpi for oral and throat infection.*

Keywords: *Kabpi, Thai traditional medicine, Antimicrobial activity*

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The bacteria infection were associated with high morbidity and mortality especially with immunocompromised patients[1]. Although many antibiotic drugs can treat infectious diseases, it can destroy many organs especially liver, kidney or cells in many organs in body. Many Thai traditional remedy and Thai medicinal plants have long been used for the treatment in infectious diseases.

A Thai traditional remedy called Kabpi (KP) has long been used for treatment oral and throat infection. It is a remedy in the Mukalok scripture, which is described in traditional Thai book called Patsatsongkraue. It is composed of four plants such as, flower of *Syzygium aromaticum* (L.) Merr. et Perry, seed of *Nigella sativa* L., bark of *Limonia acidissima* L. and rhizome of *Achasma sphaerocephalum* Holtt. There are the antimicrobial reports of some plants in Kabpi. All of parts of *Limonia acidissima* L. showed antimicrobial activity against *S. aureus* with 22-38 mm[2]. Eugenol that was isolated from flower of *Syzygium*

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aromaticum (L.) Merr. et Perry exhibited as active antimicrobial against *C. albicans* with MIC level as 0.64 mg/ml[3]. The methanolic extract of *Nigella sativa* L. seed can exhibit antimicrobial activity[4]. However, there is no research antimicrobial activity on Kabpi remedy extract and its some plant component extract. Therefore, the purpose in this study is to determine antimicrobial activity of Kabpi remedy and its plant component extracts by different extraction methods against microbial in oral and throat such as one type of bacteria *S. aureus* ATCC 25923 and one type of fungi *C. albicans* ATCC 90028.

Material and Method

Chemicals and reagents

95% ethanol (CMJ Anchor company, Thailand), Distilled water (Milford, USA), Dimethyl-sulphoxide (RCI Labscan, Thailand), Mueller-Hinton Agar (Difco, USA), Mueller-Hinton Broth (Difco, USA), Nutrient Agar (Difco, USA), Sabouraud Dextrose Agar (Difco, USA) and Resazurin (Sigma, USA).

Plant materials

Thai medicinal remedy Kabpi and its plant components used for oral and throat infection were collected from several parts of Thailand in October 2012 except *Nigella sativa* L., which was bought from India. The voucher specimen and traditional used are shown in Table 1. The voucher specimens were deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkla Province, Thailand.

Preparation of crude extracts

All plant materials were washed, sliced and dried in a hot air oven at temperature of 50°C, ground to be powder into a 40-mesh particle size powder and extracted by maceration with 95% and 50% ethanol, and boiling in water as ethanolic extract and water extract respectively. The residue from maceration with 95% ethanol and 50% ethanol were boiled in water. The plant material (300 g) and Kabpi remedy (400 g) were macerated in 95% ethanol 3 days, filtered through whatman No. 1 and the filtrate was evaporated by evaporator. The residue was macerated 2 times, combined three extracts and evaporated until stable weight. This extract obtained 95% ethanol extract. In the same procedure, its plant material and Kabpi remedy (200 g) also macerated in 50% ethanol and obtained as 50% ethanol extract. The residue of extraction by maceration on 95% and 50% ethanol 3 times were boiled

Table 1. Botanical, ethnopharmacological used and biological activity of plants which are components of Kabpi

Scientific name: family	Common name	Thai name	Voucher specimen number	Part used	Thai trditional use[11]	Biological activity
Syzygium aromaticum (L.) Merr. et Perry : Myrtaceae	Clove	Kan-phlu	SKP 123 19 01 01	Flower	Treatment of bacterial infection, stomachic, antidiarrheal, toothache	Antioxidant[9] Antimicrobial[2,7] Cytotoxic[10]
Nigella sativa L. : Ranunculaceae	Black seed, black caraway, black cumin	Thian-dam	SKP 160 14 19 01	Seed	Treatment of carminative, digestive, toothache, headache, nasal congestion, hypertension	Anti-inflammatory[11-15] Antimicrobial[4] Antioxidant[16,17] Cytotoxic[18]
Limonia acidissima L. : Rutaceae	Wood apple	Ma-kuid	SKP 166 12 01 01	Bark	Treatment of diarrhea, swelling, abscess	Antimicrobial[2,7] Anti-inflammatory[19]
Achasma sphaerocephalum Holtt. : Zingiberaceae	-	Kha-daeng	SKP 201 01 19 01	Rhizome	Treatment of menstrual disorders	-

in water, filtered and dried by lyophilizer, they obtained as residue of 95% and 50% ethanol. Dried plants material (100 g) was boiled in distilled water at boiling point for 15 min, three times, then filtrated and dried by lyophilizer. This extract is water extract.

The preparation and plant extract were calculated as percentage of yield. The crude extracts were kept at -20°C.

Determination of antimicrobial activity

Microorganisms

Bacterial strains were for testing against one type of gram positive bacteria *Staphylococcus aureus* (ATCC 25923) and one type of fungi *Candida albicans* (ATCC 90028). Bacteria was cultured in Nutrient agar (NA) at 37°C for 24 hours, while fungi was cultured in Sabouraud dextrose agar (SDA) at 37°C for 48 hours.

Preparation of inocula

Isolated colonies of one bacteria and one fungi were cultured into Mueller-Hinton broth (MHB) at 37°C for 2 hours. After that, suspension was adjusted turbidity to 0.5 McFarland standards.

Preparation of test disc

The extracts in 95% ethanol and 50% ethanol were dissolved in dimethylsulphoxide (DMSO) to a final concentration 500 mg/ml and the extracts with decoction and the residue from maceration 95% ethanol and 50% ethanol were dissolved in distilled water (Mili-Q, $\geq$18 Mega Ohm) to a final concentration of 100 mg/ml. Then prepared extracts 10 µl were applied on 6 mm sterile paper discs.

Antimicrobial assay

Disc diffusion method

The agar disc diffusion method was used to screen antimicrobial activity of the extracts by Lorian V., 1996[5]. Sterilized filter paper discs (6 mm in diameter) were impregnated with 10 µl of the extracts. Turbidity to 0.5 McFarland standards was diluted with Mueller-Hinton Broth (MHB). Then, swabbed in the inoculums suspension, removed excess fluid and swab the entire the Mueller-Hinton Agar (MHA) surface evenly in three directions with sterile cotton swabbed, leave on the plate for 3-5 minutes. After that, place the dried paper discs on to the lawn on the Mueller-Hinton Agar (MHA) with sterile forceps. Plates with microorganism and test samples were incubated at 37°C for 18-24 hours and fungi for 48 hours. Finally, measurement of inhibition zone (clear zone) around the disc was interpreted the susceptibility and resistance of the microorganism to test antimicrobial activity. The zone of inhibition (clear zone) was calculated by measuring the diameter. Positive controls were Gentamicin (Conc 1 µg) for bacteria and Amphotericin B for fungi (Conc 1 µg).

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) values were determined by microtiter plate-based assay by Sarker et al, 2007[6]. The ethanolic extracts for testing were dissolved in Dimethylsulfoxide (DMSO). Water extracts were dissolved in distilled water (Mili-Q, $\geq$18 Mega Ohm) and filtration with Millipore filter 0.22 µm (Merck Millipore, Tullagreen). After that, were prepared samples to 10 mg/ml. The culture were prepared for 18-24 hours culture of were *S. aureus* (ATCC 25923) and 48 hours of *C. albicans* (ATCC 90028). The inoculum was adjusted turbidity equal to 0.5 McFarland standard and diluted with sterile Mueller-Hinton Broth (MHB) at 1:200 to give a final concentration of 5×10^5 CFU/ml. Added 50 µl of extract solution to concentration 5, 2.5 mg/ml in sterile 96 wells microtiter plates (corning incorporated, USA). Serial two-fold dilutions was diluted the sample. Then, added 50 µl of the inoculums into sterile 96 wells microtiter plates. The plates were covered with a sterile plate sealer and plastic wrap. Then, agitated to mix were the contents of the wells using a plate shaker and incubate at 37°C, 18-24 hours for *S. aureus* (ATCC 25923) and 48 hours for *C. albicans* (ATCC 90028).

After the specified time, 10 µl of resazurin solution (blue compound, 7-hydroxy-3H-phenoxazin-3-one 10-oxide) was added at concentration 1 mg/ml into each well. Then, the plate was incubated at 37°C for 3 hours. The result was interpreted by the change of color of resazurin. MIC value is the lowest concentration of crude extract solution that can inhibit the growth of microorganism by showing a color change in sterile 96 well microtiter plates. The extract solution can inhibit microorganism by generating blue color of resazurin. In contrast, the extract solution no inhibit microorganism the rezasurin solution were changed to purple or pink color. The assay was repeated in triplicate. Positive control, negative control and viable control are included.

Statistical analysis

All data were carried out in triplicate. Values of different parameters were expressed as the mean $\pm$ standard deviation. Statistical analysis was performed using Prism Software.

Results

Kabpi remedy (KP) and four plant extracts were showed percentage of yield in Table 2. They were tested screening antimicrobial activity by disc diffusion and minimal inhibitory concentration (MIC) one type of bacteria against *S. aureus* ATCC 25923 (Table 3) and one type of fungi against *C. albicans* (Table 4). The results were found that 95% ethanol extract of KP showed antimicrobial activity against *S. aureus* and *C. albicans* (MIC = 0.625, 0.625 mg/ml respectively). The zone of inhibition against *S. aureus* and *C. albicans* ranged from 7.33 to 21.00 mm and 10.00 to 23.33 mm, respectively. Four extracts of *Syzygium aromaticum* flowers and the ethanolic extract of *Achasma sphaerocephalum* rhizomes showed the same MIC value as 0.625 mg/ml against *S. aureus*. The 95% ethanol extracts of *Syzygium aromaticum* flowers also showed the highest antifungal activity against *C. albicans* (MIC = 0.156 mg/ml). However, the extract of *Limonia acidissima* barks had no activity against with *C. albicans*.

Discussion

These results related with the previous report on study of ingredient of *Syzygium aromaticum* flowers as eugenol, it showed antimicrobial against *C. albicans* with MIC level as 0.64 mg/ml[3]. However, in this study the ethanolic extract of *Syzygium aromaticum* flowers showed higher antifungal activity than eugenol as its components in previously reported with MIC as 0.156 mg/ml. In addition, 95% ethanol extract of flowers from this plant has ever been reported on against *S. aureus* and showed high antibacterial activity against all bacterial stains the inhibition zone in range of 9 to 19 mm and it against *S. aureus* with MIC value as 1.25 mg/ml[7]. Interestingly, in this study, the different extraction method of *Syzygium aromaticum* flowers except maceration with 50% ethanol showed higher antibacterial activity against *S. aureus* than previously report (MIC values as 0.625 mg/ml). In addition, the maceration method with 95% and 50% ethanol of this plant also showed high antifungal activity against *C. albicans* with MIC values as 0.156 and 0.3125 mg/ml, respectively. There are two reports of the methanolic extract of *Nigella sativa* seeds exhibited antibacterial activity against *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus cereus* by inhibition zone = 7-9, 7-9 and >15 mm respectively[4] and the 95% ethanolic extract of *Nigella sativa* seeds exhibited antibacterial activity against *S. aureus* by MIC = 5 mg/ml[7]. This study found that the 95% ethanol extract of *Nigella*

Table 2. The percentage of yield of extracts from Kabpi remedy and plant components by different extraction methods

Extracts	% Yield				
	95% ethanol	50% ethanol	Water	Residue of 95% ethanol	Residue of 50% ethanol
Preparation of Kabpi	7.01	9.40	11.83	8.30	4.12
Syzygium aromaticum (L.) Merr. et Perry	14.88	17.05	18.06	21.45	14.25
Nigella sativa L.	4.88	6.55	10.46	9.45	4.30
Limonia acidissima L.	2.49	2.41	2.58	2.00	6.38
Achasma sphaerocephalum Holtt.	5.66	12.34	9.04	6.59	2.22

Table 3. Antimicrobial activity of extracts expressed as diameter of inhibition zones (mm) and minimal inhibitory concentration valued MIC (mg/ml). Positive control is Gentamicin (µg/ml) for *Staphylococcus aureus* (ATCC 25923) (n = 3)

Extracts	*Staphylococcus aureus* (ATCC 25923)									
	95% ethanol		50% ethanol		Water		Residue of 95% ethanol		Residue of 50% ethanol	
	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)
Preparation of Kabpi	17.67±1.16	0.625	14.33±1.16	2.50	10.67±0.58	1.250	10.67±1.53	1.250	10.00±1.00	1.250
Syzygium aromaticum (L.) Merr. et Perry	14.67±1.16	0.625	14.00±0.00	1.25	12.33±0.58	0.625	12.00±1.00	0.625	12.67±0.58	0.625
Nigella sativa L.	19.00±1.00	1.250	8.33±0.58	5	NI	-	NI	-	NI	-
Limonia acidissima L.	NI	-	7.33±0.58	>5	NI	-	NI	-	NI	-
Achasma sphaerocephalum Holtt.	21.00±1.00	0.625	NI	-	NI	-	NI	-	NI	-
Gentamicin (positive control)	Disc 22.00±0.00 (mm)				MIC 0.195 (µg/ml)					

NI = no inhibition

Table 4. Antimicrobial activity of extracts expressed as diameter of inhibition zones (mm) and minimal inhibitory concentration valued MIC (mg/ml). Positive control is Amphotericin B (µg/ml) for *Candida albicans* (ATCC 90028) (n = 3)

Extracts	*Candida albicans* (ATCC 90028)									
	95% ethanol		50% ethanol		Water		Residue of 95% ethanol		Residue of 50% ethanol	
	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)	Disc (mm)	MIC (mg/ml)
Preparation of Kabpi	16.00±1.00	0.625	13.00±0.00	5	NI	-	NI	-	NI	-
Syzygium aromaticum (L.) Merr. et Perry	23.33±1.53	0.156	18.33±1.53	0.3125	11.67±0.58	5	10.00±0.00	5	11.67±0.58	5
Nigella sativa L.	NI	-	8.33±1.53	5	NI	-	NI	-	NI	-
Limonia acidissima L.	NI	-	NI	-	NI	-	NI	-	NI	-
Achasma sphaerocephalum Holtt.	10.00±0.00	5	NI	-	NI	-	NI	-	NI	-
Amphotericin B (positive control)	Disc 21.00±0.00 (mm)				MIC 1 (µg/ml)					

NI = no inhibition

sativa L. showed greater antimicrobial activity against *S. aureus* than previously reported (inhibition zone = 19 and 13 mm and MIC as 1.25 and 5 mg/ml of this study and previously study respectively). One of report of *Limonia acidissima* L. barks extracted by a soxhlet apparatus with petroleum ether and $CHCl_3$ exhibited antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterobacter eloacae, Klebsiella erogenes, Aspergillus niger* and *Candida albicans* resulted in MIC in the range of 25-100 µg/ml[8]. This result did not agree with the previous study because MIC of all extracts of *Limonia acidissima* barks was more than 5 mg/ml. The last component of Kabpi is *Achasma sphaerocephalum* rhizomes, only its ethanolic extract showed higher antibacterial activity against *S. aureus* than antifungal activity against *C. albicans* (MIC = 0.625 and 5 mg/ml, respectively). This report is the first report on antimicrobial activity of Kabpi and *Achasma sphaerocephalum* rhizome extract. By the conclusion, the 95% ethanolic extract of Kabpi should be a continuously developed product for oral and throat infection. The active plant components of the ethanolic Kabpi are all plants except *Limonia acidissima* barks that has an astringent taste. In traditional of Thai medicine theory, it was described that the plant, which had an astringent taste, and could be astringent and would heal.

Conclusion

At the conclusion, the 95% ethanolic extract of Kabpi remedy exhibited the highest antimicrobial activity against the microbe, which was the cause of oral and throat infection such as *S. aureus* and *C. albicans* by the same MIC value (0.625 mg/ml). The active of plant ingredients for antimicrobial activity are *Syzygium aromaticum* flower, *Nigella sativa* seed and *Achasma sphaerocephalum* rhizome, but *Limonia acidissima* bark may be astringent and would heal. However, Kabpi remedy can be concluded that it can be used for oral and throat infection against oral infection and support using Thai traditional medicine. The product development of Kabpi remedy should be continuously investigated as being the drug of choice for sore throat such as mouth wash or lozenges. Kabpi remedy should also be continued to investigate the antimicrobial compounds of this remedy and its plant components.

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Potential conflicts of interest

None.

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การศึกษาฤทธิ์ต้านเชื้อแบคทีเรียและเชื้อราของตำรับยากัปปีที่ใช้รักษาโรคติดเชื้อในช่องปากและลำคอและสมุนไพรเดี่ยวในตำรับ

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ภูมิหลัง: ตำรับยากัปปีเป็นตำรับยาที่ใช้รักษาโรคติดเชื้อในช่องปากและลำคอมาอย่างยาวนาน โดยประกอบไปด้วยสมุนไพร 4 ชนิด ดังต่อไปนี้คือ ดอกกานพลู (Syzygium aromaticum (L.) Merr. et Perry), เมล็ดเทียนดำ (Nigella sativa L.), เหง้าข่าแดง (Limonia acidissima L.) และเปลือกต้นมะขวิด (Achasma sphaerocephalum Holtt.)

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ในการต้านเชื้อแบคทีเรียและฤทธิ์ต้านเชื้อราของสารสกัดจากตำรับยากัปปี และสมุนไพรเดี่ยวในตำรับ

วัสดุและวิธีการ: สกัดสารด้วยวิธีการหมักด้วยเอทานอล 95% และ 50% ทำแห้งด้วยเครื่องปั่นเหวี่ยงภายใต้ระบบสูญญากาศ (evaporator) สกัดสารด้วยการต้มน้ำ ทำแห้งด้วยเครื่องทำแห้งแบบแช่แข็ง (lyophilizer) สำหรับกากที่เหลือจากการหมักด้วยเอทานอล 95% และ 50% นำมาต้มน้ำเพื่อสกัดอีกครั้ง และทำแห้งด้วยเครื่องทำแห้งแบบแช่แข็ง (lyophilizer) การศึกษาในขั้นตอนแรกนำสารสกัดไปทดสอบฤทธิ์การต้านเชื้อแบคทีเรียและเชื้อราด้วยวิธี disc diffusion โดยทดสอบกับเชื้อแบคทีเรียแกรมบวก 1 ชนิด Staphylococcus aureus และเชื้อรา 1 ชนิด Candida albicans เมื่อทดสอบด้วยวิธี disc diffusion พบว่าสารสกัดสมุนไพรมีฤทธิ์ต้านเชื้อจึงนำสารสกัดสมุนไพรนั้นมาทดสอบด้วยวิธี microtiter plate-based assay เพื่อหาค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อได้ (MIC)

ผลการศึกษา: สารสกัดด้วยเอทานอล 95% ของตำรับยากัปปีมีฤทธิ์ในการต้านเชื้อ S. aureus และ C. albicans (MIC = 0.625, 0.625 มิลลิกรัม/มิลลิลิตร) มีค่าเส้นผ่าศูนย์กลางในการยับยั้งเชื้อ (inhibition zone) ของสารสกัดทั้งหมดอยู่ในช่วง 7.33-23.33 มิลลิเมตร สารสกัดด้วย เอทานอล 95% ของข่าแดง มีค่าเส้นผ่าศูนย์กลางในการยับยั้งเชื้อ (inhibition zone) S. aureus กว้างที่สุดโดยมีค่าเส้นผ่าศูนย์กลางในการยับยั้งเชื้อ (inhibition zone) 21.00 มิลลิเมตร และมีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อ (MIC) = 0.625 มิลลิกรัม/มิลลิลิตร สารสกัดด้วยเอทานอล 95% ของกานพลูมีฤทธิ์ที่ดีในการยับยั้งเชื้อ C. albicans มีค่าเส้นผ่าศูนย์กลางในการยับยั้งเชื้อ (inhibition zone) 23.33 มิลลิเมตร และมีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อ (MIC) = 0.156 มิลลิกรัม/มิลลิลิตร

สรุป: จากผลการศึกษาทำให้ทราบว่าตำรับยากัปปี และสมุนไพรเดี่ยวในตำรับมีฤทธิ์ในการต้านเชื้อแบคทีเรีย และเชื้อราซึ่งสามารถนำผลการศึกษาที่ได้มาสนับสนุนถึงประสิทธิภาพในการรักษาโรคติดเชื้อในช่องปากและลำคอได้ต่อไป