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ผลการต่อต้านการเจริญของเชื้อแบคทีเรียก่อโรคในสัตว์โดยน้ำมันหอมระเหย

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บทคัดย่อ: การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพการต้านการเจริญของเชื้อแบคทีเรียของน้ำมันหอมระเหยชนิดต่างๆที่สกัดมาจากพืชท้องถิ่นของประเทศไทย น้ำมันหอมระเหยจำนวน 8 ชนิดที่สกัดจากตะไคร้ ตะไคร้หอม มะกรูด ส้มมะงั่ว โหระพา พริกไทยดำ กะเพรา และแมงลัก ถูกนำมาทดสอบเพื่อหาสมบัติการต้านการเจริญของเชื้อก่อโรค คือ *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium และ *Pseudomonas aeruginosa* ด้วยวิธี agar-disc diffusion ผลการทดลองแสดงให้เห็นว่าน้ำมันหอมระเหยจากพืชต่างชนิดกันจะแสดงสมบัติในการต้านการเจริญของเชื้อทดสอบที่แตกต่างกันออกไป พบว่าน้ำมันหอมระเหยจากตะไคร้มีแนวโน้มในการยับยั้งเชื้อทดสอบได้ดีกว่าน้ำมันหอมระเหยอื่นๆ แต่ไม่สามารถพบสมบัติในการยับยั้งเชื้อทดสอบจากน้ำมันหอมระเหยจากกะเพราและแมงลัก ค่าความเข้มข้นน้อยที่สุดที่สามารถฆ่าเชื้อทดสอบทุกชนิดของน้ำมันหอมระเหยจากตะไคร้เมื่อทดสอบด้วยวิธี Microbroth dilution พบว่ามีค่าในช่วง 125-500 ppm ผลจากการศึกษาครั้งนี้ชี้ให้เห็นถึงประสิทธิภาพของการยับยั้งการเจริญของเชื้อแบคทีเรียก่อโรคของน้ำมันหอมระเหยที่สกัดจากพืชชนิดต่างๆ

คำสำคัญ: น้ำมันหอมระเหย พืชท้องถิ่นของไทย ประสิทธิภาพในการยับยั้งการเจริญของแบคทีเรีย

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Antimicrobial Effect against Animal Pathogenic Bacteria by Essential Oils

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Abstract: The objective of this study was to investigate the antimicrobial effect of essential oils from local Thai plants. Eight essential oils of *Cymbopogon citratus* Stapf. (Lemongrass), *Cymbopogon nardus* Rendle (Citronella grass), *Citrus hystrix* DC. (Leech lime), *Citrus medica* Linn (Citron), *Ocimum basilicum* L. (Sweet basil), *Piper nigrum* L. (Black pepper), *Ocimum sanctum* L. (Holy basil) and *Ocimum basilicum* L.f. var *citratum* Back (Hairy basil) were evaluated for their antimicrobial properties by agar-disc diffusion technique against pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa*. The results showed that various degrees of antibacterial activity were found from different essential oils. The essential oil from *Cymbopogon citratus* Stapf. showed higher tendency to inhibit tested pathogens when compared with other essential oils used in this study. However, the inhibitory effects of essential oils from *Ocimum sanctum* L. and *Ocimum basilicum* L.f. var *citratum* Back were not observed. Minimum Bactericidal Concentration (MBC) of *Cymbopogon citratus* Stapf. essential oil by microbroth dilution test was ranged between 125-500 ppm for all tested pathogenic bacteria. The results showed the antimicrobial activities of essential oils from different plants against pathogenic bacteria.

Keywords: Essential oil, Thai plant, Antimicrobial efficiency

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Introduction

Due to the tremendous demand for meat and dairy products, the livestock production has been growing rapidly worldwide. The Food and Agricultural Organization (FAO) estimated that there are more than 4.0 billion domestic animals including cattle, pigs, sheep, goats and horses which employ over 1.3 billion people (Brooks-pollock *et al*., 2015). Although the productions in livestock are needed and would make a lot of fortune, bacterial

infection is still one of the problems in livestock industry which causes economic losses. To treat bacteria-infectious disease in animals, antibiotics have been wildly used without doubt. However, the use of antibiotics in animal feeds has been raising various hidden problems such as antibiotic-resistant bacteria, antibiotic residue and serious health problems coming over (Hong *et al*., 2012).

Herbal plants have been drawing a lot of attentions as an alternative to as antibiotic in animal feeds because of their naturally phytochemical components, demonstrating antimicrobial activity (Jarriyawattanachaikul *et al*., 2016). Among these phytochemical substances, produced in plants, essential oils are one of the most popular candidates owing to its distinct biological functions (Celikel and Kavas, 2008). Essential oils have been reported to possess beneficial effects to intestinal microflora, digestive enzymes and immune system (Hong *et al*., 2012). In addition, the essential oils have also been reported to have anti-microorganism properties such as bacteria, parasites and fungi (Talbert and Wall, 2012; Katiki *et al*., 2012; Singh *et al*., 2009; Oussalah *et al*., 2007; Fichi *et al*., 2007). Therefore, this study was aimed to determine the *in-vitro* antimicrobial activity of selected essential oils, extract from local plants in Thailand, against pathogenic bacteria in animals.

Materials and Methods

Preparation of essential oils

Essential oils extract of *Cymbopogon citratus* Stapf. (Lemongrass), *Cymbopogon nardus* Rendle (Citronella grass), *Citrus hystrix* DC. (Leech lime), *Citrus medica*Linn (Citron), *Ocimum basilicum* L. (Sweet basil), *Piper nigrum* L. (Black pepper), *Ocimum sanctum* L. (Holy basil) and *Ocimum basilicum* L.f. var *citratum* Back (Hairy basil) were prepared by hydro-distillation method. Briefly, plant matters were packed in round bottle flask with sufficient amount of water and subsequently brought to boil. Then the vapor mixture of essential oil and water was condensed by indirect cooling from the condensing part. To separate water from essential oil, the essential oil was dried over sodium sulfate anhydrous and store at 4°C until use.

Screening for antimicrobial activity

To screen for the antimicrobial activity of each essential oil, *Staphylococcus aureus* (TISTR 746), *Escherichia coli* (TISTR 117), *Salmonella* Typhimurium (TISTR 1469) and *Pseudomonas aeruginosa* (TISTR 357) were used in this study. A single colony of bacteria was grown in nutrient broth, 16-18 hours at

37°C to obtain the bacterial concentration of $1x10^8$ CFU. The nutrient agar plate was covered with 100 µl of bacteria culture. The 6 millimeter-sterilized paper discs with 10 µL of essential oil to impregnate were placed on the nutrient agar plate. The plates were incubated at 37ºC for 24 hours to observe antimicrobial activity. Disc with of oxytetracycline (50 µg) and distilled water were used as positive and negative control, respectively. All tested were done in triplicate and antimicrobial activity was evaluated by measurement the diameter of inhibition zone in millimeter (mm.).

Microbroth Dilution Assay

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were evaluated by using Resazurin Microtiter Assay Plate method (Rahman *et al*., 2004). One hundred microliters of nutrient broth were pipetted into each well in 96-well plate. A two-fold dilution was prepared the essential oil in various concentrations. One hundred microliters of the bacteria were added to the mixture of essential oil. The concentration used of each essential oil in this study was in the range of 0.12-500 ppm. The 96-well plates were incubated at 37°C for 24 hours to obtain MIC. MIC is defined as the lowest concentration of the essential oil with no visible growth of indicator strain. After that, 30 µL of 0.02% resazurin was added. The plates were then incubated at 37°C for 24 hours to obtain MBC. MBC is defined as the lowest concentration of essential oil with no color change of resazurin, indicating 95% of inoculation was killed.

Results

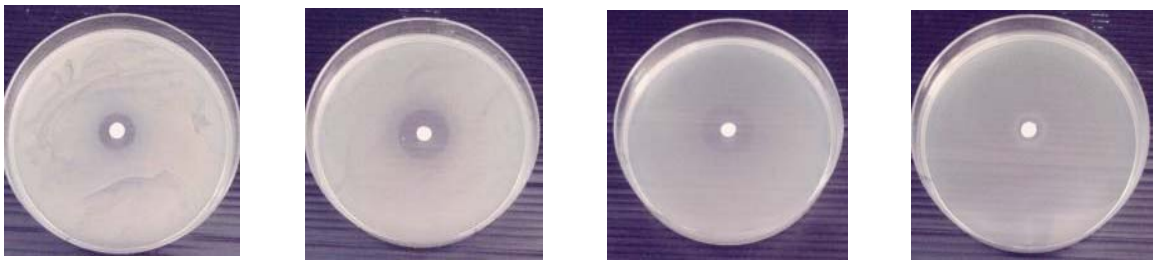
Screening for antimicrobial activity

The results of antimicrobial activity showed in Table 1. There was found that essential oils from different plant species possessed various antimicrobial activity. Of 8 essential oils tested, 6 essential oils revealed their antimicrobial activities over tested bacteria. On the other hand, another two essential oils, extracted from *Ocimum sanctum* L. and *Ocimum basilicum* L.f. var *citratum* Back did not show any antimicrobial activity against bacteria. The highest inhibition zone (32.06±0.99 mm.) was found in the present of essential oil from *Cymbopogon citratus* Stapf. against *E. coli* (Table1 and Figure 1). It was about 1.5 times higher than control (20.13±0.21 mm.). Oppositely, the lowest inhibition zone was observed when testing the *Ocimum basilicum* L. essential oil with *S. aureus* (7.70±0.49 mm.) (Table1).

Table 1 *In-vitro* antimicrobial activity of essential oils

Source of Essential oil	Indicator strain			
	S. aureus	*E. coli*	*S.* Typhimurium	*Ps. aeruginosa*
Cymbopogon citratus Stapf.	14.14±0.52	32.06±0.99	29.26±0.93	7.95±0.32
Cymbopogon nardus Rendle	11.69±0.18	30.99±0.22	27.97±0.94	9.01±0.58
Citrus hystrix DC.	10.63±0.82	10.31±0.88	11.50±0.75	10.47±0.52
Citrus medica Linn	8.08±0.39	8.20±0.16	9.17±0.83	10.32±0.86
Ocimum basilicum L.	7.70±0.49	11.85±0.82	11.08±0.73	8.42±0.78
Piper nigrum L.	10.54±0.23	11.82±0.50	8.77±0.28	9.98±0.74
Ocimum sanctum L.	-	-	-	-
Ocimum basilicum L.f. var *citratum* Back	-	-	-	-
Distilled water	-	-	-	-
Oxytetracycline (50 μg/disc)	20.27±0.45	20.13±0.21	29.71±0.95	11.61±0.14

Data expressed as mean of triplicate ± standard deviation (SD) including the disc diameter (6 mm.). All values were rounded to two decimal places. "-" indicated no inhibition.



a b c d

Figure 1 Inhibition zone of essential oil from *Cymbopogon citratus* Stapf. Against *S. aureus* (a), *E. coli* (b), *S.* Typhimurium (c) and *Ps. aeruginosa* (d)

Microbroth dilution assay

MIC and MBC values of essential oils were presented in Table 2 and Figure 2. The data showed that MIC values of all essential oils tested in this study towards *S. aureus*, *E. coli*, *S.* Typhimurium and *Ps. aeruginosa* were found as 500, 250, 500 and 125 ppm, respectively. Nevertheless, the MBC values of tested essential oils were diverse, with noted that the essential oil extracted from *Cymbopogon citratus* Stapf. was the most active essential oil. It was because this

essential oil had the lowest MBC values against *S. aureus* and *S.* Typhimurium which were 500 and 500 ppm, respectively. However, the MBC values of the essential oil from *Cymbopogon citratus* Stapf. against *E. coli* and *Ps. aeruginosa* were the same as those of essential oils from other plants (250 and 250 ppm, respectively).

Discussion

Agar-disc diffusion results indicated the variety of antimicrobial activity among essential oils against pathogenic bacteria. The differences between antimicrobial activities might be due to various major and minor components, present in essential oil of each plant. Components, synthesized in each plant were claimed to be responsible for their alteration in antimicrobial activity of essential oils (Oussalah *et al*., 2007). These plant components were influenced by geographical origin and environmental condition (Celikel and Kavas, 2008). However, the broth dilution results were not correlated with agar-disc diffusion test, showing that the inhibition zones of most essential oils against *Ps. aeruginosa* seemed to be lower than the inhibition zones, observed in other indicator strains. The difference between these two methods might come from the problem of the diffusion property of components in essential oils, resulting in agar-disc diffusion technique was less reliable than broth dilution assay. These contradictory results between agar-disc diffusion and dilution assay were also reported by Yousef and Tawil (1980) when they studied on the antimicrobial activities of 22 essential oils. Rios *et al*. (1988) concluded on the conflict between these two methods that the agar-disc diffusion technique was not acceptable for the samples which were difficult to soluble water or non-polar samples. In addition, there is no relation between diffusion power and antimicrobial activity as well as there is no relationship between MIC values and inhibition diameter. However, the agar-disc diffusion method was suitable for screening in the term of the small size sample used and the possibility to test many compounds against a single microorganism.

To determine MIC and MBC with the most precise technique and suitable for non-polar samples such as essential oils, a dilution method was recommended (Clark *et al*., 1981; Miski *et al*., 1983; Rios *et al*., 1988). Therefore, the microbroth dilution assay was employed in this study to evaluate the strength of antimicrobial activity. The result showed that the MIC and MBC values of essential oil from *Cymbopogon citratus* Stapf. towards *S. aureus*, *E. coli*, *S.* Typhimurium showed the same result (500, 250 and 500 ppm, respectively). It demonstrated that this

Table 2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of each essential oil

Source of Essential oil		Indicator strain			
	MIC/MBC (ppm)	*S. aureus*	*E. coli*	*S.* Typhimurium	*Ps. aeruginosa*
Cymbopogon citratus Stapf.	MIC	500	250	500	125
	MBC	500	250	500	250
Cymbopogon nardus Rendle	MIC	500	250	500	125
	MBC	>500	250	>500	250
Citrus hystrix DC.	MIC	500	250	500	125
	MBC	>500	500	>500	250
Citrus medica Linn	MIC	500	250	500	125
	MBC	>500	500	>500	250
Ocimum basilicum L.	MIC	500	250	500	125
	MBC	>500	500	>500	250
Piper nigrum L.	MIC	500	250	500	125
	MBC	>500	500	>500	250

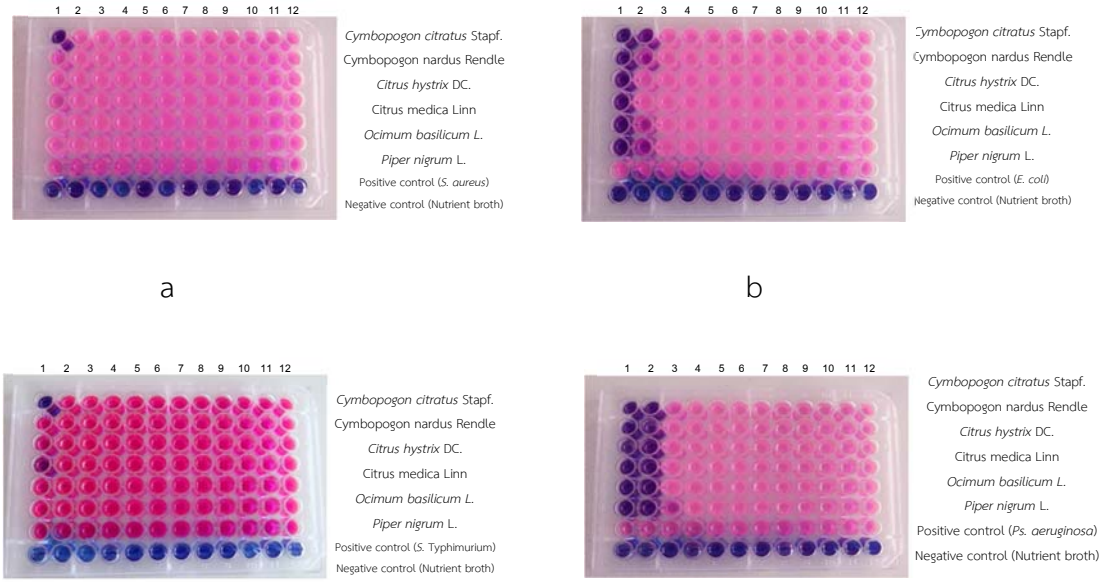


Figure 2 MBC result of essential oils against *S. aureus* (a), *E. coli* (b), *S.* Typhimurium (c) and *Ps. aeruginosa* (d). The concentrations of each essential oil were in the range of 500-0.12 ppm; 500 ppm (1), 125 ppm (2), 62.5 ppm (3), 31.3 ppm (4), 15.6 ppm (5), 7.81 ppm (6), 3.91 ppm (7), 1.95 ppm (8), 0.98 ppm (9), 0.49 ppm (10), 0.24 ppm (11) and 0.12 ppm (12).

essential oil was inhibitory and bactericidal at a single concentration. For *Ps. aeruginosa*, it seemed to be the most sensitive bacteria towards *Cymbopogon citratus* Stapf. as noticed by the lowest MIB and MBC values. Hence, the susceptibility of microorganisms by essential oil from *Cymbopogon citratus* Stapf. was *Ps. aeruginosa* > *E. coli.* > *S. aureus* and *S.* Typhimurium. This data imply that the gram-negative bacteria were more vulnerable than gram-positive bacteria towards essential oils. This data did not agree with the report from Suwanpugdee *et al*. (2012), reporting that the essential oils from lemongrass and citronella grass could successfully inhibit *S. aureus* and followed by *E. coli*. Nevertheless, comparison on the susceptibility of bacteria might vary. It was not only because of the antimicrobial agents in essential oils, but also the variation of tested microorganisms (Kamazen *et al*., 2012; Oussalah *et al*., 2007; Hammer *et al*., 1999). Another possible factor might contribute the different results was the type of media used to test for antimicrobial activity. Using different kinds of media might differ in bacteria growth condition. For example, the media used by Suwanpudee *et al*. was Tryptic Soy Broth while Nutrient Broth was used in this study. These aforementioned factors can contribute the result differently. However, it could be said that the promising antimicrobial property of essential oils, especially the essential oil from *Cymbopogon citratus* Stapf. could be possibly applied in animal treatments, as an alternative to antibiotic for animal infectious treatment. Yet, the toxicity and safety issue of using this essential oil would need to be considered.

Conclusion

To summarize, the essential oils had a potential to inhibit and inactive both gram-negative and gram-positive bacteria. Their antimicrobial activities were different, depending on their phytochemical components. However, the essential oil from *Cymbopogon citratus* Stapf. seem to be the most active to inhibit the tested microorganisms in *in-vitro* condition.

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