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ผลของฤทธิ์ต้านเชื้อแบคทีเรียของสารสกัดหยาบมะรุมต้านเชื้อ *Staphylococcus aureus* จากเต้านมอักเสบในโคนม

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บทคัดย่อ: *Staphylococcus aureus* เป็นเชื้อก่อโรคที่มีความรุนแรงมาก ซึ่งเป็นสาเหตุของโรคเต้านมอักเสบทั้งแบบแสดงอาการและแบบไม่แสดงอาการทางคลินิกในโคนม โรคเต้านมอักเสบเป็นโรคที่ทำให้เกิดความสูญเสียทางเศรษฐกิจโดยส่งผลกระทบต่อผลผลิตน้ำนม และคุณภาพน้ำนมที่ลดลง การรักษาด้วยยาปฏิชีวนะมักจะไม่ประสบความสำเร็จ เป็นเพราะว่าเชื้อ *S. aureus* สามารถต้านทานต่อยาปฏิชีวนะได้โดยการแทรกซึมเข้าไปยังเนื้อเยื่อของต่อมสร้างน้ำนม และที่มากไปกว่านั้นการใช้ยาปฏิชีวนะในการรักษาโรคเต้านมอักเสบมักทำให้เกิดการตกค้างของยาปฏิชีวนะในน้ำนมและผลิตภัณฑ์นม ดังนั้นสมุนไพรจึงเป็นสิ่งที่น่าสนใจอีกแนวทางเลือกหนึ่งของการรักษาที่ได้จากแหล่งธรรมชาติ มะรุมเป็นพืชสมุนไพรไทยมีสรรพคุณทางยาอย่างกว้างขวาง และรักษาเต้านมอักเสบในโคนมได้ การวิจัยนี้จึงมีวัตถุประสงค์ประเมินฤทธิ์ต้านเชื้อแบคทีเรียของสารสกัดหยาบมะรุมต่อเชื้อ S. *aureus* and *S. aureus* ATCC 25923 ทำการศึกษามะรุมโดยใช้ใบสด ดอกสด ฝักสด ใบแห้ง และฝักแห้งนำมาสกัดเป็นสารสกัดหยาบด้วยตัวทำละลายคือเอทานอล แล้วนำสารสกัดหยาบทั้งหมดมาศึกษาฤทธิ์ต้านเชื้อแบคทีเรียโดยวิธี disc diffusion และเจือจางความเข้มข้นของสารสกัดหยาบตามลำดับโดยรายงานผลเป็นค่าความเข้มข้นต่ำสุดที่ยับยั้งเชื้อได้ (MIC) และค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อได้ (MBC) ผลการศึกษาพบว่าใบสดที่สกัดด้วยเอทานอลให้ผลผลิตของสารสกัดหยาบสูงที่สุดคือ 9.30 เปอร์เซ็นต์ และเมื่อนำสารสกัดหยาบของใบสดที่ความเข้มข้น 20 มิลลิกรัมต่อแผ่น มาทดสอบด้วยวิธี disc diffusion พบว่าต้านเชื้อ *S. aureus* โดยมีขนาดวงใส 12.83±1.47 มิลลิเมตร และต้านเชื้อ *S. aureus* ATTC 25923 โดยมีขนาดวงใส 14.67±0.52 มิลลิเมตร ค่าความเข้มข้นต่ำสุดที่ยับยั้งเชื้อได้ (MIC) และค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อได้ (MBC) ต่อเชื้อ *S. aureus* อยู่ในช่วง 4-64 มิลลิกรัมต่อมิลลิลิตร และเชื้อ *S. aureus* ATCC 25923 อยู่ในช่วง 32-64 มิลลิกรัมต่อมิลลิลิตร การศึกษาวิจัยในครั้งนี้จึงบ่งชี้ว่าสารสกัดหยาบของมะรุมสามารถหยุดการเจริญเติบโตของเชื้อ *S. aureus* ที่ก่อโรคเต้านมอักเสบในโคนมได้

คำสำคัญ: ฤทธิ์ต้านเชื้อแบคทีเรีย มะรุม *Staphylococcus aureus* เต้านมอักเสบในโคนม

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The Effect of *Moringa oleifera* Lam. Extracts on Antibacterial Activity against *Staphylococcus aureus* from Bovine Mastitis

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Abstract: *Staphylococcus aureus* is a contagious pathogen as a cause of clinical and subclinical mastitis in dairy cows. The economic disease is affected to decrease milk productivity and milk quality. The antibiotic treatment is not successfully because *S. aureus* can resistance and penetration in tissue of the mammary gland. In addition, the used of antibiotic treatment has been causing antibiotic residue in milk and milk products. Therefore, traditional herbs are interested in a new alternative treatment from natural resources. *Moringa oleifera* Lam. is the Thai herb that has a wide range of medical properties for treatment of bovine mastitis. This research aimed to evaluate antibacterial effect of *M. oleifera* Lam. crude extracts against *S. aureus* and *S. aureus* ATCC 25923. Fresh of leaves, flowers and pod pulp and powder of leaf and pod pulp of *M. oleifera* Lam. were extracted by ethanol. All crude extracts were tested for antibacterial activity using by disc diffusion and broth dilution methods as reported by the minimum inhibitory concentration (MIC) values and the minimum bactericidal concentration (MBC) values. The highest yield by ethanol extraction of fresh leaves was 9.30%. The disc diffusion method result showed that the inhibition zones of crude extract from fresh leaves at 20 mg/disc against *S. aureus* and *S. aureus* ATCC 25923 were 12.83±1.47 mm. and 14.67±0.52 mm., respectively. The MIC and MBC values of *S.aureus* were range 4-64 mg/ml and 32-64 mg/ml, respectively. This research indicates that the *M. oleifera* Lam. crude extracts prohibited the growth of *S. aureus* from mastitis in dairy cows.

Keywords: Antibacterial activity, *Moringa oleifera* Lam., *Staphylococcus aureus*, Bovine Mastitis

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Introduction

Bovine mastitis is an economic loss disease for dairy farmers. This disease leads to an inflammation of the mammary gland that frequently develops in teat and teat canal (Douglas and Robert, 2005; Petrovski et al., 2006)

Bovine mastitis results in reduction of milk production and milk quality. The disease is divided into clinical and subclinical mastitis. Clinical mastitis is characterized by abnormal milk, clots or flakes milk, udder swelling, redness and systemic signs including fever, pain and anorexia (Forsback et al., 2009; Blowey, 2010). Subclinical mastitis shows non visible change in the milk or the udder whereas bacteria are presented in the secretion of milk and the number of somatic cell increase (Erskine, 2001; Harmon, 1994). Both clinical and subclinical mastitis cause an increase in milk somatic cell count and changes in milk composition (Forsback et al., 2009). *Staphylococcus aureus* is the most important causative microorganism and contagious pathogen which it causes both clinical and subclinical intramammary infection (Dego, 2002; Roberson et al., 1994). The antibiotic treatment has been incriminated for resistance in bacteria isolated from treated animals, other animals within the herd, food derived from cattle for human consumption (Pantosti et al., 2007) and a cause of antibiotic residue in milk product (Barkema et al., 2006; NMC, 1996). *M. oleifera* Lam., a common name is Maroom in Thai, is the medical herb. It has a wide range of medical properties; antibacterial properties anti-inflammatory, anti-hyperglycemic, anti-oxidant, anti-tumor and anti-cancer (Ademiluyi et al., 2018; Gopalakrishnan et al., 2016). All parts of *M. oleifera*; leaves, flowers, pod and pod pulp, provide vitamins and nutrients (Zaku et al., 2015; Abdull et al., 2014).

Therefore, this experiment focused on the effective parts of plant extraction, and studied antibacterial activity of *M. oleifera* Lam. crude extract against *S. aureus*. The results of this study can be applied for treatment of the bovine mastitis in the future.

Materials and methods

Preparation of ethanol extracts

M. oleifera Lam. was obtained from Chumphon province in the single batch collection. Fresh *M. oleifera* Lam. were cleaned with water and cut for leaves, pod pulp and flowers. Then, they were splitted into 2 parts. The first part, there were fresh leaves, flowers and pod pulp. The second part were powder from leaves and pod pulp. They were dried at 50°C in the hot air oven for 24 hr and then finely ground into a powder by cutting mill machine. After that, all parts were extracted with 95% ethanol (1:10) and macerated at room temperature for 7 days. The extracts were hand-squeezed through a thin cloth and a filter paper (Whatman No.1), respectively. To obtain the crude extracts, ethanol solvent were removed using a rotary evaporator. The crude extracts were kept at -20°C until use.

Isolation of S. aureus from bovine mastitis

One ml of milk sample was pipetted into 10 ml of Tryptic Soy Broth (TSB) with 1% pyruvate and 10% sodium chloride, and incubated at 37°C for 24-48 hr. Then, touching 2-3 loops from TSB and streaking onto Baird-Parker agar (BPA) and incubated at 37°C for 24-

48 hr. Colonies of *S. aureus* are circular, smooth, convex, and gray to jet-black and surrounded by opaque zone.

Identification of S. aureus

S. aureus was confirmed by coagulase positive test. After that, the colonies of *S. aureus* were transferred into small tubes containing 2 ml of Brain Heart Infusion Broth (BHI) and incubated at 37°C for 18-24 hr. The coagulase test was performed by adding 20 μl of solution from BHI into 200 μl of coagulase plasma solution. Then, it was incubated at 37°C for 24 hr. *S. aureus* was also confirmed by mannitol salt agar (MSA).

Screening of crude extract for antibacterial activity by disc diffusion test

The experimental designed with factorial in completely randomize design (CRD) consists of two factors. These factors of the crude extract were a concentration in 6 levels and a part of plant in 5 kinds. The disc diffusion test was performed using sterile 6 mm-diameter filter paper discs. The discs were prepared using 20 μl of each crude extract diluted in the solvent (dimethyl sulfoside; DMSO for ethanol extracts) to concentrations of 1,000, 800, 400, 200, 100 and 50 mg/ml, respectively. Thus, the concentration of each disc contained 20, 16, 8, 4, 2 and 1 mg of crude extract, respectively. All discs were dried at room temperature overnight. Preparation of bacteria, at least three isolated colonies of the same morphological type were selected from blood agar culture. The top of each colony was touched with a sterile wire loop and transferred to a tube containing 5 ml of Mueller-Hinton broth (MHB). The broth was incubated at 37°C (usually 3-5 h) until the turbidity of the 0.5 McFarland standard using the McFarland densitometer. A 150 μl of the 0.5 McFarland suspensions were transferred to 5 ml of Mueller-Hinton agar (MHA), the final inoculum on the agar was approximately 10^6 CFU/ml. The discs were placed on the surface of the MHA and incubated at 37°C 18-24 hr. Pure DMSO and 95% ethanol were used as negative controls while amoxicillin-clavulanic acid (30 μg, Oxoid) and cephalotin (30 μg, Oxoid) were used as positive controls. The disc diffusion test was determined by measuring the diameter of the inhibition zone. The experiments were performed in 6 replicates and the means of the diameters of the inhibition zones were calculated.

Determination of the minimum inhibitory concentration (MIC) of crude extracts by the modified resazurin assay

The modified resazurin assay was performed using sterile 96 well plates (Greiner bio-one, Germany) for determining MIC. Fifty μl of MHB was added into all wells. Fifty μl of the initial concentration of crude extracts prepared at 1,024 mg/ml were added into the first well. After that, 50 μl from their serial dilutions were transferred into nine consecutive wells. The negative controls were DMSO and 95% ethanol. Finally, 50 μl of the 0.5 McFarland suspensions were added into all wells. The plates were incubated at 37°C for 18-24 hr. After incubation,

bacterial growth was evaluated by adding 50 µl of resazurin solution; 5 mg of one resazurin tablet in 50 ml of sterile distilled water, and the plates were extended incubation for one hour. The color change was then assessed visually from purple to pink and compared to the control plate. The lowest concentration at which color change was recorded as the MIC values that inhibited the bacteria growth.

Determination of the minimum bactericidal concentration (MBC) of crude extracts by the mannitol salt agar

The MBC was determined by touching the loop from each well of MIC plate and streaking it on MSA. The plates were incubated at 37°C for 18-24 hr. The unchange media color is defined as the MBC; the lowest concentration, that kill the bacteria. The yellow color of media shows *S. aureus* growth.

Statistical analysis

The statistical analysis was compared the inhibition zone was performed using ANOVA with Duncan's multiple comparison test. Inhibition zones were expressed as mean ± SD.

Results

The extraction of *M. oleifera* Lam. from various plant parts were performed. The yields (%) of crude extracts from *M. oleifera* Lam. calculated on the initial weight, 200 g of the fresh leaves, flowers, pod pulp, the powder of leaf and pod pulp. The results showed the weight of crude extracts of those were 18.61, 11.11, 9.72, 11.07 and 10.91 g, respectively (data not shown). The yields of crude extracts of *M. oleifera* Lam. from the fresh leaves, flowers, pod pulp, the powder of leaf and pod pulp were 9.30, 5.55, 4.86, 5.53 and 5.45, respectively (Table 1). At the ratio of ethanol and part of plant, we found that the highest yield of crude extract was fresh leaves.

The antibacterial activity of crude extracts against *S. aureus* depended on the crude extract concentrations and the parts of plant as shown in significantly at *p- value* ≤ 0.01. At the higher concentration of crude extract gave the better result than of at the lower concentration as shown in the diameters of inhibition zone (Table 2). The result showed that at 20 mg/disc combination with part of fresh leaves had the highest antibacterial activity which was 12.83 mm of inhibition zone diameter. Whereas, at 1 mg/disc combination with part of fresh pod pulp and pod pulp powder had the lowest antibacterial activity which was 6.00 mm of inhibition zone diameter.

Besides the leaves, it found that the flowers had an antibacterial effect which shown diameter of inhibition zone was 9.00 mm, at 20 mg/disc concentration. Another interesting feature, the pod pulp had ability to inhibit growth of *S. aureus* which at 20 mg/disc concentration, the inhibition zone diameter, was 8.17 mm.

The similar results on the concentration of crude extracts and the parts of plant had an antibacterial activity effect against *S. aureus* ATCC 25923 (Table 2).

Table 1 The yields of crude extracts from *M. oleifera* Lam.

Crude extracts	Extract yields (%)
The ethanol extract of fresh leaves	9.30
The ethanol extract of fresh flowers	5.55
The ethanol extract of fresh pod pulp	4.86
The ethanol extract of leaf powder	5.53
The ethanol extract of pod pulp powder	5.45

The negative controls showed no result of inhibitory effect. By contrast, the positive controls of amoxicillin-clavulanic acid and cephalotin showed that there was a substantial zone of inhibition which were at the range of 24-29 mm.

The MIC values of the extracts were determined by the modified resazurin microtiterplate. The DMSO and 95% ethanol used as controls showed no inhibitory effect. The MIC values were range 4-64 mg/ml for *S. aureus* and 8-128 mg/ml for *S. aureus* ATCC 25923. The result of MIC values showes in Table 3.

The MBC values of the extracts were determined by touching the loop from each well of MIC plate and streaking it on MSA and were incubated at 37°C for 18-24 hrs. The DMSO and 95% ethanol were negative controls which show no sign of *S. aureus* survival. The MBC values were range 32-64 mg/ml for *S. aureus* and 16-1,024 mg/ml for *S. aureus* ATCC 25923. The result of MBC values shows in Table 3.

Discussion

The *M. oleifera* Lam. is an interesting medical herb for an alternative treatment on mastitis in dairy cows. Fouad et al. (2019) reported on an antibacterial activity against both positive and negative bacteria. *S. aureus* is one of the most important causative agents of mastitis (Dego, 2002; Douglas and Robert, 2005). This research was interested in this bacteria due to antibiotics resistance dilemma causing by the agent.

The ethanol is the most effective solvent using for extraction of medical plant on antibacterial activity (Fouad et al., 2019; Opara et al., 2015). As the result, the ethanol extract of fresh leaves provided the best result. This is similar to the experiment use ethanol extraction of *M. oleifera* Lam. leaves against *S. aureus* isolated from camel milk (Fouad et al., 2019). The ethanol extraction of *M. oleifera* Lam. leaves had an effect on variety strains of *S. aureus* (Fouad et al., 2019; Kalpana et al., 2013). The other parts; the flowers and pod pulp, of the crude extracts with ethanol solvent also presented the antibacterial activity. This research also demonstrated individual leaf and pod pulp powder. The antibacterial activity of the extract from pod pulp powder had better result than that of the extract from leaf powder. The use of

Table 2 Antibacterial activity, measured as mean (± SD) of inhibition zone (mm), of *M. Oleifera* Lam. crude extracts against *S. aureus* and *S. aureus* ATCC 25923

Concentration of crude extracts (mg/disc)	Parts of plant	Inhibition zone (mm)	
		S. aureus	*S. aureus* ATCC 25923
1	fresh leaves	8.33±1.86^{bcd}	9.33±0.52^{ef}
	fresh flowers	6.33±0.52^{gh}	6.00±0.00^{k}
	fresh pod pulp	6.00±0.00^{h}	6.00±0.00^{k}
	leaf powder	6.33±0.52^{gh}	6.67±0.52^{ijk}
	pod pulp powder	6.00±0.00^{h}	6.00±0.00^{k}
2	fresh leaves	8.33±1.86^{bcd}	10.50±0.84^{d}
	fresh flowers	6.50±0.55^{fgh}	6.33±0.52^{jk}
	fresh pod pulp	6.00±0.00^{h}	6.00±0.00^{k}
	leaf powder	6.33±0.52^{gh}	7.17±0.75^{ghijk}
	pod pulp powder	8.67±2.07^{bc}	6.33±0.52^{jk}
4	fresh leaves	8.67±2.07^{bc}	10.67±0.52^{cd}
	fresh flowers	7.67±0.52^{bcdefg}	8.33±2.07^{fg}
	fresh pod pulp	6.00±0.00^{h}	6.67±1.03^{ijk}
	leaf powder	6.67±1.03^{efgh}	7.33±0.82^{ghij}
	pod pulp powder	6.67±1.03^{efgh}	6.67±0.52^{ijk}
8	fresh leaves	8.00±1.55^{bcdef}	10.50±0.84^{d}
	fresh flowers	7.67±1.86^{bcdefg}	9.33±2.25^{ef}
	fresh pod pulp	6.67±0.52^{efgh}	7.00±0.89^{hijk}
	leaf powder	6.33±0.52^{gh}	7.33±0.52^{ghij}
	pod pulp powder	7.00±0.00^{defgh}	6.67±0.52^{ijk}
16	fresh leaves	9.00±1.55^{b}	10.00±0.89^{de}
	fresh flowers	8.50±1.64^{bcd}	11.67±1.37^{c}
	fresh pod pulp	7.33±0.52^{cdefgh}	8.00±0.89^{gh}
	leaf powder	6.67±0.52^{efgh}	7.67±0.52^{ghi}
	pod pulp powder	8.33±1.03^{bcd}	8.00±0.63^{gh}
20	fresh leaves	12.83±1.47^{a}	14.67±0.52^{a}
	fresh flowers	9.00±1.41^{b}	13.50±1.22^{b}
	fresh pod pulp	8.17±1.47^{bcde}	9.17±1.33^{ef}
	leaf powder	6.67±0.52^{efgh}	7.67±0.52^{ghi}
	pod pulp powder	8.67±0.52^{bc}	7.67±1.03^{ghi}
Concentration of crude extracts		**	**
Part of plant		**	**
Concentration * Part of plant		**	**

In column, means followed by a different letter are significantly different by $DMRT_{0.05}$

** are significantly difference at probability ≤ 0.01

Table 3 The MIC/MBC values of *M. oleifera* Lam. crude extracts against *S. aureus* and *S. aureus* ATCC 25923

M. oleifera Lam. Crude extracts	*S. aureus*		*S. aureus* ATCC 25923	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
The fresh leaves	16	32	32	64
The fresh pod pulp	32	32	16	128
The fresh flowers	4	32	8	64
The leaf powder	64	64	128	1,024
The pod pulp powder	32	32	16	16

the powder extraction by ethanol also found in many researchs (Kalpana et al., 2013; Tirado et al., 2018). There was a report on using combination of leaf, stem and seed powder against *S. aureus* showed significant results (Tirado et al., 2018).

In conclusion, the crude extracts of *M. oleifera* Lam. inhibited the growth of *S. aureus* from bovine mastitis. The antibacterial activity of crude extracts from *M. oleifera* Lam. was solely based on *in vitro*. The widest inhibition zone of crude extract against *S. aureus* derived from fresh leaves. As our results leads to further study on *in vivo* activity of the crude extracts of *M. oleifera* Lam. in bovine mastitis and product development of teat dipping solution in dairy farm.

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