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Antibacterial activity of *Rafflesia kerrii* Meijer extracts against hospital isolates of methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

Rafflesia kerrii Meijer has been used as a traditional medicine for treatment of many diseases and for prolonging life. However, there has been little evidence to support the pharmacological effect. This study, therefore, aims to evaluate the antibacterial activity of *R. kerrii* Meijer crude extract from three different parts, i.e., perigone lobe, central disk and process to against six *S. aureus* strains include *S. aureus* ATCC 25923 and five clinical isolates of methicillin-resistant *S. aureus* (MRSA) from hospital. The antibacterial activity was determined using disc diffusion and broth microdilution methods. The results showed that all three part extracts from *R. kerrii* Meijer exhibited potent antibacterial activity against to *S. aureus* ATCC 25923 and five clinical isolates of MRSA from hospital. The perigone lobe extract exhibited the best antibacterial effect on *S. aureus* ATCC 25923 and the MRSA clinical isolate no. 1, 2, 3 and 5 followed by the central disk extract with MIC of 5 and 10 mg/ml, respectively. However, the extracts had no bactericidal effect on all tested bacteria, suggesting that the extracts had more inhibitory properties than killing activity. The results reveal that *R. kerrii* Meijer crude extracts show interestingly pharmacologic benefits for the development of a new alternative medicine.

Keywords: *Rafflesia kerrii* Meijer, antibacterial activity, *S. aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* (MRSA)

1. Introduction

Infectious diseases are major public health problems all over the world. Multidrug-resistant (MDR) is a major serious public health problem worldwide, making the treatment ineffective and nosocomial infections, consequently leading to spending long periods of the treatment and increasing the cost of treatment [1, 2]. In Thailand, the incidence of MDR bacteria has also increased [3, 4, 5]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a gram-positive bacterium that becomes resistant to many antibiotics. MRSA is a prominent human pathogen that is known for causing skin infections, as well as hospital-acquired pneumonia, osteomyelitis and abscesses, and that tends to cause serious infection in the burn patients with high mortality [6]. MRSA strains are resistant not only to β-lactam antibiotics, but also to fluoroquinolones and other families of antibiotics [7]. Therefore, alternative antimicrobial agents from plants are interesting to develop for a new antimicrobial drug.

Rafflesia kerrii Meijer is the largest flower in Thailand. The local Thai name is Bua Phut or Bua Tum [8]. *R. kerrii* Meijer is parasitic flowering plants of the genus *Tetrastigma*. The flower is 70-80 cm in diameter and weighs about 10 kg. Bua Phut is found in the rainforest of southern Thailand especially in Chumphon, Ranong and Surat Thani provinces, with the largest population in the Khao Sok National Park. The local people in Malaysia, Indonesia, and Thailand belief that flower buds boiled with water or soaked in medical wine can help to restore the uterus of post-natal's woman to a normal condition, curing diarrheal diseases, relieving fever and backache, and stimulating sexual activity [9, 10]. Literature reviews show that Bua Phut contains several bioactive compounds such as hydrolysable tannins and a phenylpropanoid glycoside [11]. Furthermore, a previous research indicates that the *R. kerrii* Meijer extract has several pharmacological effects of antibacterial, antioxidant, antimutagenicity, antityrosinase and anticancer activities against epidermoid carcinoma cells [12, 13, 14, 15]. However, no previous research has studied the pharmacological effect from separate parts of *R. kerrii* Meijer. This study is a first paper to present a preliminary investigation of the antibacterial activity of *R. kerrii* Meijer extract from 3 different parts of flower bud against the

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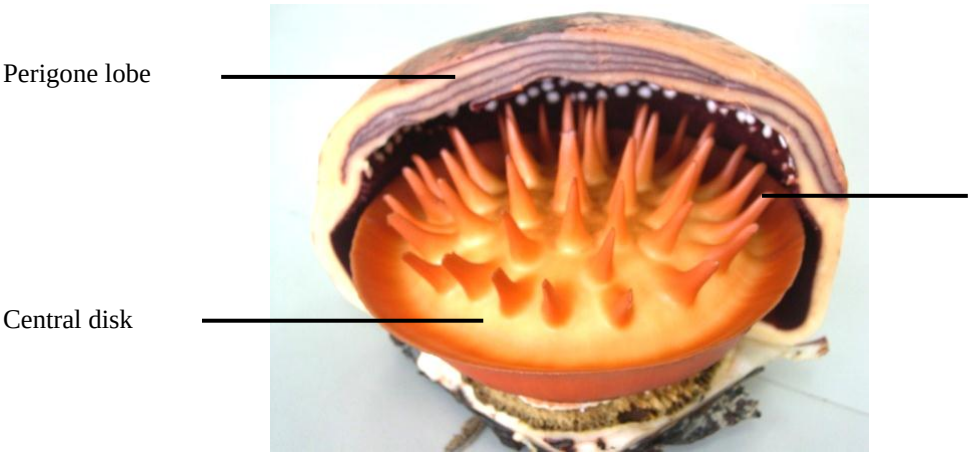

Figure 1 Section of *Rafflesia* flower

clinical isolates of MRSA compared with the reference strain *S. aureus* ATCC 25923.

2. Materials and methods

2.1 Plant materials and preparation of extracts

R. kerrii Meijer was collected in September 2008, from Chumphon province, Thailand, and kept at room temperature during transportation. *R. kerrii* Meijer was washed with clean water and cut into 3 separate parts, i.e., perigone lobe, central disk and process (Figure 1). Each part was cut into small pieces, dried under the sun baked in a 60^{o}C oven for 24 h, and powdered. The powder was proceeded extraction with 95% ethanol (w/v:1/5) for 10 days at room temperature and filtering through filter paper. Rotary evaporator was used to evaporate ethanol from the extract. The extract was stored in refrigerator at 4°C until experiment was conducted.

2.2 Bacteria cultivation

The five clinical isolates of MRSA in this study were kindly provided by the Queen Sirikit National Institute of Child Health, Bangkok, Thailand. The antibiotic resistance profiles of the MRSA isolates consist of Erythromycin, Fosfomycin, Gentamicin, Oxacillin, Penicillin G, Trimethoprim/ sulfamethoxazole and Vancomycin. The reference strain, *S. aureus* ATCC 25923 was obtained from the Department of Microbiology, Faculty of Public Health, Mahidol University, Thailand.

All bacterial strains were cultured on Nutrient agar (Difco, USA) at 37^{o}C for 24 h, suspended in Nutrient broth and adjusted to the concentration equivalent to the McFarland No.0.5 (approximately 1.5 x 10^{8} CFU/ml) [16].

2.3 Antibacterial activity test

1) Disc diffusion method

Antibacterial screening test was conducted using the disc diffusion method. Disc diffusion test was performed using sterile 6 mm-diameter filter paper discs (Whatman, UK). The sterile discs were impregnated with 20 µl Bua Phut extracts which were first dissolved in 95% ethanol, then diluted with distilled water to concentrations of 100 mg/ml and 50 mg/ml, respectively, and filter sterilized through a 0.45 µm filter. The amount of extract used were 1 mg/disc and 2 mg/disc. The impregnated filter discs were dried at 60°C for 30 min and allowed to cool down at room temperature before used.

Disc diffusion method was performed according to the method of Bauer *et al*. [17]. Briefly, bacterial suspensions were adjusted turbidity to a 0.5 McFarland standard and then spread evenly on the surface of Mueller Hinton agar (MHA) (Difco, USA) using a sterile swab. Penicillin (10 µg/ml) was used as a positive control. Filter discs impregnated with 20 µl of distilled water were used as negative controls and then incubated at 37°C for 24 h. The experiments were performed in triplicate. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the discs and interpreted according to the Clinical and Laboratory Standards Institute [18]. The results were expressed as mean ± SD.

2) Broth microdilution method

Minimum inhibitory concentration (MIC) assay

Minimal inhibitory concentration (MIC) of *R. kerrii* Meijer crude extract was determined by using a microdilution technique in a 96-well microplate according to the method of Langfield *et al*. [19]. Fifty µl of trypticase soy broth (Merck, Germany) was added into the 2nd well until the 12th well. One hundred µl of crude extract was added into the 1st well and serial two-fold dilutions were made down to the 11th well. Fifty µl. of the last mixture was discarded. The 50 µl. of the bacterial suspension (1.5 x 10^{8} CFU/ml) was added into each well, mixed and incubated at 37^{o}C for 24 h. The final concentration of each extract was therefore ranged from 0.156 to 20 mg/ml. Penicillin (10 µg/ml) was used as a positive control and 95%

Table 1 Yield of *R. kerrii* Meijer extraction

Part of *R. kerrii* Meijer	Fresh weight (g)	Dry weight (g)	Extract weight (g)	% Yield of extract
Perigone lobe	5,860.0	254.9	30.0	11.8
Central disk	3,910.0	68.0	15.0	22.1
Process	430.0	6.5	1.2	18.8

ethanol (0.156- 20 mg/ml) was used as a negative control. Finally, 40 µl of 4 mg/ml *p*-iodonitrotetrazolium violet (Sigma-Aldrich, USA) was added into each well and incubated for an additional 2 h. [20]. MIC was determined as the lowest sample concentration at which no red color (signifying live growth) appeared. The experiment was performed in triplicates.

Minimum bactericidal concentration (MBC) assay

The cultures of MIC assay that did not show turbidity were taken to spread on Mueller-Hinton agar (Difco, USA). The plates were incubated at 37^{o}C for 24 h. MBC is defined as the lowest concentration in MIC that did not show bacterial growth. The experiment was performed in triplicates.

3. Results and discussion

3.1 Extraction of *R. kerrii* Meijer

Three parts of *R. kerrii* Meijer, i.e. perigone lobe, central disk and process were extracted and fractionated with 95% ethanol. The crude extracts are semisolid and sticky with dark brown color. The yields as compared to the dry weights of the perigone lobe, central disk and process extracts were 11.77, 22.05 and 18.76%, respectively (Table 1). The central disk extract had highest percent yield, compared to other extracts.

3.2 Antibacterial activity of *R. kerrii* Meijer crude extracts

The antibacterial activities of all three extracts against six *S. aureus* strains including *S. aureus* ATCC 25923 and five clinical isolates of methicillin-resistant *S. aureus* (MRSA) from hospital are shown in Table 2. The results of antibacterial activity of all three extracts by disc diffusion method revealed that *S. aureus* ATCC 25923 was sensitive only to perigone lobe and central disk extracts at the concentration of 1 mg/disc with the inhibition zone of 8 and 7 mm and was sensitive to 2 mg/disc of the perigone lobe, central disk and process extracts with the inhibition zones of 10.5, 8 and 9.5 mm, respectively. All clinical isolates of MRSA were sensitive to all three extracts with the inhibition zone ranging from 8 to 11 mm and 9 to 13 mm at 1 mg/disc and 2 mg/disc of extracts, respectively. The perigone lobe extract showed potent inhibitory activity to *S. aureus* ATCC 25923 and the clinical isolates of MRSA no. 1, 2, 3 and 5 with MIC of 5 mg/ml.

Interestingly, all three extracts from *R. kerrii* Meijer flower bud showed antibacterial activity against all tested clinical isolates of MRSA, although all tested clinical isolates of MRSA were multidrug resistant, which was consistent with previous research by Chuangchot *et al.* [14]. They reported that the whole flower buds of *R. kerrii* Meijer extract had antibacterial activity against five clinical isolates of MRSA with the MIC range of 1.56–2.34 mg/ml. The results showed that the perigone lobe extract exhibited the highest effective activity against clinical isolates of MRSA. The process extract exhibited the lowest antibacterial activity, compared to other extracts. However, the extract had no bactericidal effect on all tested bacteria, suggesting that the extracts had inhibitory activity greater than bactericidal activity.

The active compounds responsible to the antibacterial activity in *R. kerrii* Meijer extracts were not investigated in this study. However, the chemical compounds of *R. kerrii* Meijer have been reported by Kanchanapoom *et al*. [11]. They found that this plant contains five compounds; 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucopyranoside, 1,2,6-tri-*O*-gallol-*β*-D-glucopyranoside, 1,4,6-tri-*O*-galloyl-*β*-D-glucopyranoside, 1,2,4-tri-*O*-galloyl-*β*-D-glucopyranoside and phenylpropanoid glucoside. The first four compounds are hydrolyzable tanins and the last one is syringin. Similarly, a previous research study reported that the member of *Rafflesiaceae* family is enriched with tannin [8] as well as *R. hasseltii* Suringar which consists of five compounds of nicotine, caffeine, leucoanthocyanin (tannin), catechin and phenolic acid [21]. The antimicrobial mechanisms of tannins include direct binding to proteins and forming tannin-protein complex with hydrogen bond formation between the phenolic hydroxyl groups of the tannin and the carbonyl groups of the peptide bond [22, 23, 24, 25, 26]. The complex formation leads to the inactivation of the protein and loss of function by inactivating microbial adhesions, inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation or iron deprivation [27, 28]. Interestingly, a previous study found that the perigone lobe extract showed high total phenolic content of 381.74 ± 4.68 GAE mg/g extract [29]. In addition, other studies reported that *R. kerrii* Meijer extract had high total phenolic content of 669.7 ± 38.6 mg TAE/g [14]. The antibacterial activity has been attributed to the presence of phenolic compounds in the extract. The mechanisms responsible for the antimicrobial activity of phenolics include an adsorption and a disruption of microbial membranes, interaction

Table 2 Antibacterial activity of *R. kerrii* Meijer crude extracts against methicillin-resistant *S. aureus* (MRSA) as compared to *S. aureus* ATCC 25923 measured by disc diffusion, MIC and MBC assays

Bacterium strain		Part of *R. kerrii* Meijer extract	Zone of inhibition (mm)[a] by disc diffusion assay			MIC (mg/ml)	MBC (mg/ml)
			1 mg	2 mg	Antibiotic control		
S. aureus ATCC 25923		perigone lobe	8.0 ± 0	10.5 ± 0	35.0 ± 0[b]	5	>20
		central disk	7.0 ± 0	8.0 ± 0	35.0 ± 0	20	>20
		process	0	9.5 ± 0	35.0 ± 0	20	>20
Methicillin Resistant *S. aureus* (MRSA)*	Isolate 1	perigone lobe	11.0 ± 0	11.5 ± 0	0	5	>20
		central disk	10.0 ± 0	11.0 ± 0	0	10	>20
		process	10.0 ± 0	11.0 ± 0	0	NT	NT
	Isolate 2	perigone lobe	10.0 ± 0	11.0 ± 0	0	5	>20
		central disk	10.0 ± 0	10.0 ± 0	0	20	>20
		process	8.0 ± 0	9.0 ± 0	0	NT	NT
	Isolate 3	perigone lobe	9.5 ± 0	10.0 ± 0	0	5	>20
		central disk	9.0 ± 0	10.0 ± 0	0	10	>20
		process	9.0 ± 0	9.0 ± 0	0	NT	NT
	Isolate 4	perigone lobe	11.0 ± 0	13.0 ± 0	0	10	>20
		central disk	10.0 ± 0	11.0 ± 0	0	20	>20
		process	9.0 ± 0	10.0 ± 0	0	NT	NT
	Isolate 5	perigone lobe	10.0 ± 0	10.0 ± 0	0	5	>20
		central disk	10.0 ± 0	10.0 ± 0	0	10	>20
		process	9.0 ± 0	10.0 ± 0	0	NT	NT

Values are presented as mean ± S.E.M (N=3)
*tested for drug resistance by Queen Sirikit National Institute of Child Health.
[a] Diameter of inhibition zone including diameter of disc 6 mm (tested at a volume of 20 µl/disc).
[b] Penicillin (10 µg/ml) was used as a positive control.
NT = not tested

with enzymes and substrates and metal ion deprivation [30, 31]. So this is not surprising why the perigone lobe extract exhibits the antibacterial activity higher than other extracts.

To the best of our knowledge, no previous research study has studied the antibacterial activity from separate parts of *R. kerrii* Meijer. Here is the first report to show that the perigone lobe extract has potent antibacterial activity against the clinical isolates of MRSA than other parts, the activity should be due to high content of tannin compounds that need to investigate further.

4. Conclusions

In summary, the result revealed that all three extracts from *R. kerrii* Meijer had antibacterial activity against both the reference strain of *S. aureus* ATCC 25923 and the clinical MRSA isolates from hospital. The results also indicated that the perigone lobe extract was highest effective in antibacterial activity, compared to other extracts. Confirmation by broth microdilution method also supported these findings. However, the bactericidal activity was not found, suggesting that the extracts had inhibitory activity greater than bactericidal activity. Further studies are needed to identify the active compounds from separate part of *R. kerrii* Meijer.

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