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Anti-Methicillin resistant *Staphylococcus aureus* Activity of *Carallia brachiata* L. Extract

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In Viet Nam, antibiotic is an important class of drugs because infectious diseases in Viet Nam is the top ratio of infections and death. Methicillin-resistant *Staphylococcus aureus* (MRSA) accounted for 82.1% of all isolates of *S. aureus* as the cause of death related to hospital-acquired pneumonia. Developing towards our ongoing studies of screening anti-*Staphylococcus aureus* activity of several local plants, we herein revealed the anti-MRSA activity of the extract of *Carallia brachiata* L. cultivated in Vietnam. The dilution method was performed to confirm the MIC value of fractions that contained anti-MRSA activity. The capacity of *Carallia brachiata* L. extract to inhibit MRSA's hemolytic properties was determined by culturing MRSA on blood agar plates. The fractions from the ethanol *Carallia brachiata* L. extract were collected using liquid-liquid phase separation. According to the results, the minimum inhibitory concentration (MIC) value of ethyl acetate *Carallia brachiata* L. extract fraction against MRSA is approximately 2.5 mg/mL. The hemolytic capacity of MRSA is inhibited by ethyl acetate *Carallia brachiata* L. extract fraction. The potency of anti-MRSA activity of *Carallia brachiata* L. extract is reported for the first time.

1. Introduction

The genus *Staphylococcus* has around 40 species, three of which are recognized as pathogens in medicine: *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. *S. aureus* is an opportunistic microorganism that attacks open wounds and individuals with weakened immune system. Bacteria usually parasitize the noses, throats, and skin of humans and animals (Taylor and Unakal, 2022).. *S. aureus* was extremely sensitive to antibiotics, when penicillin was introduced for clinical use. It was only a few years later that penicillin-resistant strains of *S. aureus* were discovered. Penicillin-resistant *S. aureus* (PRSA) accounts for approximately 90 percent of all *S. aureus* strains isolated, while MRSA ranges from 30 to 50 percent. Methicilin, a semisynthetic penicillin capable of penetrating bacterial cell walls, was developed in 1960 (Ministry of health portal, 2015). Methicillin-resistant MRSA exhibits two common resistance mechanisms: overexpression of β-lactamases and a change in the normal structure of penicillin-binding proteins (PBPs). The methicillin-resistant mechanism with the expression of the PBP2a protein is encoded by the *mecA* gene in the operon *mec* region, which is present in clinical MRSA. Operon *mecA* has two regulatory factors, mecI proteins (which are inhibitors) and mecR1 (which is a transmembrane activator). mecI and mecR1 are functionally analogous to blaI and blaR1: upon binding to β-lactam, the intracellular mecR1 region is released to degrade mecI and permit transcription of *mecA* gene. In the majority of clinical strains, the presence of the PBP2a protein is the best popular mechanism of action in the emergence of antibiotic resistance. MRSA has a mechanism of resistance to β-lactam antibiotics such as methicillin and penicillin, making MRSA infections difficult to treat (Otto, 2012). It seems that identifying the therapy for treating MRSA is necessary.

Xang Ma or Xang Ma Che/Sang Ma is called in Viet Nam, and the scientific name is *Carallia brachiate* L. Adult *Carallia brachiata* L. trees can reach up to a height of 33 m and a diameter of 210 cm. They are located in lowland forests and forest swamps from Madagascar to Australia, along with China. The bark is reddish-brown with patches of gray. Inside the yellow-brown bark is yellow wood. Peduncles are 4 mm in length. The

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inflorescences are 1 cm in length. The fruit is 4 mm long, ovate, and open at the apex. In Cambodia, Laos and Vietnam, the plant is used to treat scabies (Ho, 1999). In Malaysia, the leaves are used to make tea and to treat blood infections; the bark is used to relieve itching (Wiart, 2006). The plant is known for its range of megastigman compounds such as 3-hydroxy-5,6-epoxy-β-ionol-3-O-β-apiofuranosyl-(1→6)-β-glucopyranoside, flavonoids, hygroline, and tannins (Krishnaveni et al., 2009). The extracts, fractions or essences extracted from *Carallia brachiate* L. have been studied. Extracts and fractions exhibit antioxidant, anti-inflammatory, and wound-healing properties (Krishnaveni et al., 2009). The antibacterial activity, in general, of *Carallia brachiate* L. extract has not been reported. The purpose of this study is determined anti-MRSA activity of fractional extracts of *Carallia brachiate* L. The anti-*Staphylococcus aureus* activity of a few plants including *Cratoxylum cochinchinense*, *Carallia brachiata*, *Syzygium glomeratum, Grewia asiatica* L. has disclosed (Thanh et al., 2020*)*, as the results of a deliberated screening process. In this report, the anti-MRSA activity of the extract of *Carallia brachiate* L. was unprecedentedly investigated via MIC value and hemolytic capacity.

2. Methods and materials

2.1 Preparation of materials

Methicillin-sensitive *Staphylococcus aureus* strain (MSSA) ATCC 6538 used as a control strain, Methicillin-resistance *Staphylococcus aureus* strain (MRSA) ATCC 33591 provided by ATCC, USA. Both strains, were cultured on Tryton Soy Broth (Himedia, India) and Tryton Soy agar (Himedia, India), incubated at 37 °C for 18 – 24 h in an incubator (DaiHan, Korea).Fresh biomass of Xang Ma (*Carallia brachiata* L.*)* (leaves and young branches) was collected from Febuary to May, in Thu Dau Mot City, Binh Duong Province, 11000’33.02" North; 106039’00.37" East at an altitude of 11 m ± 3 m, pressure of 0.999 atm.

2.2 Moisture measurement

To determine moisture, placing a pre-weighed amount of herbs (5-10 g) in a weighing cup with appropriate lid. The weighing cup needs to be of the appropriate size so that the added medicinal layer is no more than 5 mm thick. Place the cup containing the medicinal herbs with an opened lid in the drying cabinet and dry at a temperature of 105 ℃ for 1 h Transfer the cup to a dehumidifier until cool, cover, and weigh. Repeat the process until the difference between the 2 masses does not exceed 0.5 mg. The moisture (%) of specimens was calculated according to the following Eq(1). In the formula, moisture (%) represents the moisture content of specimens; m1 the mass of herbs before drying (g); m2 the mass of herbs after drying, in grams (Ministry of health portal, 2018)

$$moisture\ (\%) = \frac{m1 - m2}{m1} \times 100\ \% \quad (1)$$

2.3 Total ash content determination

Total ash is the inorganic material remaining after a medicinal herb has been completely burned. The procedure was conducted in a 35 mm porcelain crucible that had been red-heated, cooled, and weighed. Weigh is determined as approximately 1-5 g of cut medicinal herbs into a crucible. Burn lightly and then gradually increase the temperature so that the medicinal herbs burn out. Place the cup in the oven at a temperature of 300 ºC until a constant mass is obtained. Weigh the medicinal herbs once they are entirely inorganic and allow them to cool in a dehumidifier. Total ash content of samples was calculated according to the following formula Eq (2). In the formula Eq (2), X (%) represents Total ash content, calculated as a percentage; a the weight of cup containing ash in grams; b the weight of cup empty in grams; c the weight of samples in grams; A the moisture, calculated as a percentage (Ministry of health portal, 2018).

$$X\ (\%) = (a - b) \times \frac{100\ \%}{(c - c \times A)} \quad (2)$$

2.4 Collection of crude ethanol *Carallia brachiata* extract

Plant samples were collected in the morning. After collecting, samples were washed with tap water to remove dirt and washed again with distilled water. Samples were dried naturally in the laboratory, at about 30 ºC ± 2 ºC. Plant specimens were pureed in a mass blender after drying (the sample volume remains constant after 2-3 d of weighing). The medicinal powder was extracted by soaking with 99.5 º ethanol (ratio of 1 g of powder to 10 mL of ethanol) for a period of 24 h at a temperature of 50 ºC, shaken at 100 rpm by a thermostatic shaker. The extract was collected and filtered through Whatman filter paper, removing residues. The resulting filtrate was dried at 30 ºC using a rotary evaporator. After centrifugation, the crude ethanol extract of *Carallia brachiata* was placed in a drying cabinet at 50 ºC until a constant mass was obtained. This extract was stored in the refrigerator at 4 ºC for later use (Ingle et al., 2017).

2.5 Collection of the fraction *Carallia brachiata* extract

The crude ethanol extract of *Carallia brachiata* was dissolved in methanol and water with a volume ratio of 1:1; the extract was completely dissolved by an ultrasonic whisk within 3 min. The solution was then processed with n-hexane and ethyl acetate using liquid-liquid phase separation. First, the crude ethanol extract solution was separated by n-hexane solvent at a 1:1 (v/v) ratio, yielding the n-hexane fraction, before it was dried with Na_2SO_4. The remaining solution was separated by ethyl acetate solvent at a ratio of 1:1 (v/v), yielding the ethyl acetate fraction, which was then dried with Na_2SO_4. Extract fractions of n-hexane and ethyl acetate were dried at 30 °C using a rotary evaporator to remove all solvents, obtaining *Carallia brachiata* extract fractions. The extract fractions in 100 % DMSO, referred to as the original solutions, were stored in a refrigerator at 4 °C until use. The original solutions were diluted five times (20 % DMSO) for experiments when studying antibacterial activity. The efficiency of collecting extract fractions was calculated using the following formula Eq (3). In the formula, E The efficiency of collecting extract fraction, calculated as a percentage; m (extract fraction) represents the weight of extract fraction in g; m (crude ethanol extract) the weight of crude ethanol extract in g

$$\mathrm{E}\ (\%) = \frac{\mathrm{m\ (extract\ fraction)}}{\mathrm{m\ (crude\ ethanol\ extract)}} \mathrm{x}100\% \quad (3)$$

The efficiency of phase separation was calculated using the following formula Eq (4) (Phung, 2007). In the formula, E The efficiency of phase separation, calculated as a percentage; $E_{\text{n-hexane separation}}$ represents the efficiency of collecting n-hexane fraction, calculated as a percentage in formula Eq (3); $E_{\text{etyl acetate separation}}$ represents the efficiency of collecting ethyl acetate fraction, calculated as a percentage in formula Eq (3)

$$E_{\text{phase separation}}\ (\%) = E_{\text{n-hexane separation}} + E_{\text{etyl acetate separation}} \quad (4)$$

2.6 Determination of MRSA resistance activity and the combination ability with cefoxitin by dilution method

The minimum inhibitory concentration (MIC) is defined as the lowest concentration that inhibits bacterial growth at an observable level (Bộ, 2015). The MIC value of *Carallia brachiata* extracts against MRSA was determined using the serial dilution method on 96-well plates. Using 96-well culture plates, each well was supplemented with the following components: 90 µL of Mueller-Hinton liquid medium (MHB), 10 µL of bacterial culture media with a concentration of 10^6 CFU/mL, and 100 µL of fractional extracts dissolved in 20 % DMSO. The final volume in each well was 200 µL. Two-fold serial dilutions using the fractional *Carallia brachiata* L. extracts were carried out on a 96-well plate, with wells set up in accordance with the experiment setup (presented in the results section). The following types of wells are included in the control group: wells containing only MHB; wells containing MHB with bacterial culture media; wells containing MHB with bacterial culture media and DMSOs; and wells containing MHB with bacterial culture media and antibiotics. The wells were aerobically incubated at 37 ºC for 24 h in a cell culture incubator. After the incubation period, visual indicators and control well observations indicated the wells with no bacterial growth. From these wells, the well with the lowest concentration of extract in the serial dilutions was determined to have the MIC value. 150 µL of extract in the well was spread over 3 Mueller-Hinton agar (MHA) plates, with 50 µL of solution per plate. Afterwards, these plates were incubated for 24 h at 37 ºC in a cell culture incubator. After the incubation period, if bacteria growth was not present or the density of bacterial cells on the plate was less than 300 CFU/mL, the selected concentration of the fractional extract was determined to be the minimum inhibitory concentration (MIC) (Wayne, 2010). The experiment was repeated 3 times (each experiment was repeated on three 96-well plates) (Mai et al., 2020). The synergistic interactions between the plant extracts and cefoxitin against MRSA were quantified by the fractional inhibitory concentration (FIC) index. A series 2-fold dilution was used for both the fractional *Carallia brachiata* L. extracts (from 5 mg/mL to 0.156 mg/mL) and cefoxitin (from 1.024 mg/mL to 0.004 mg/mL). The mixing volume ratio was 1:1. The FIC index was calculated with Eq (5).

$$FIC\ Index\ = \frac{MIC\ of\ antibiotic\ in\ combination}{MIC\ of\ antibiotic\ alone} + \frac{MIC\ of\ plant\ extract\ in\ combination}{MIC\ of\ plant\ extract\ alone} \quad (5)$$

Where FICI <0.5 is synergistic, FICI from 0.5 to 0.75 is partially synergistic, FICI from 0.76 to 1 is additive effect and FICI >2 is antobonistic effect (Kuok et al., 2017).

2.7 Determination of hemolytic toxin inhibition capacity of Carallia brachiate L. extracts against MRSA strain ATCC33591

The MRSA ATCC33591 strain exhibits hemolytic capacity in sheep's blood agar media. Two strains of bacteria were cultured on blood agar plates for 24 h at 37 ºC and 5 % CO_2. The absence of hemolysis would indicate that the ability of the two strains to produce hemolytic toxins has been inhibited (Sethupathy et al., 2017). The ethyl acetate *Carallia brachiate* L. extract fraction was chosen to test its ability to inhibit the MRSA strain's

hemolytic activity on blood agar media. In this experiment, fractional extracts were added to the MRSA culture medium over a 24-hour period. The selected test tubes are then incubated for 24 h at 37 ºC in the cell culture incubator, after which they are transferred to blood agar plates and incubated for another 24 h at 5 % CO_2 in the cell culture incubator. The results were recorded after 24 h.
The experiment was repeated 3 times, and the results were presented as averages ± standard deviations. Results were statistically processed using STATGRAPHICS Centurion XV (Statpoint Technologies, USA) (Statgraphics Centurion, 2006).

3. Results and discussion

3.1 *Carallia brachiata* L. extract's anti-MRSA activity.

The preliminary analysis results of moisture and ash content were presented in Table 1. In general, different herbs have different total ash content, but the value must not be higher than 24 % (Appendix 9.8, Vietnam Pharmacopoeia Volume 2) (Bộ, 2018). *Carallia brachiata* L. has a total ash content that meets the requirements for total ash content in medicinal herbs.

Table 1: Moisture and total ash content of plant specimens

Plant names	Moisture (%)	Total ash content (%)
Carallia brachiate L.	62,27±0,09	15,74±0,11

Using the disc diffusion method, we found that a crude ethanol extract of *Carallia brachiate* L. was effective against MRSA (Figure 1). In particular, the average size of the sterile ring surrounding the well containing crude ethanol *Carallia brachiata* L. extract on MRSA was 11.5±0.5 (mm). It is worth mentioning that the MRSA resistance activity of crude ethanol *Carallia brachiata* L. extract have not been previously reported; these findings is complementary to previous reports of the its antioxidant and wound-healing benefits (Wiart, 2006). The collection of two fractional extracts from ethanol *Carallia brachiata* L. extract are practiced.

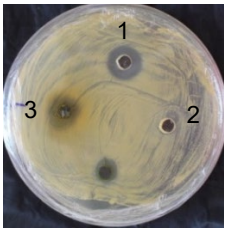

Figure 1: Anti-MRSA activity of Carallia brachiata extract fractions. Well (*1*)*: vancomycin (0,5 mg/mL) (positive control), Well* (*2*)*: n-hexane fraction, Well* (*3*)*: Ethyl acetate fraction*

The efficiency of the fractional extraction process from crude ethanol *Carallia brachiata* extract was shown in Table 2. The extracted fractions were investigated for antibacterial activity by disc diffusion method (Figure 1).

Table 2: Efficiency of extraction process

Plant names	Solvent	Efficiency of collecting extract fractions (%)	Efficienct of phase separation (%)
Carallia brachiata L.	n-hexane	8,82±0,09	20,41±0.17
	Ethyl acetate	11,59±0,08	

The results indicated that fractional ethyl acetate extract was more effective against bacteria than fractional n-hexane extract. The fractional ethyl acetate extract was chosen to determine MIC values on 96-well plates using the dilution method. In Table 3, the MIC value of the ethyl acetate fraction was 2.5 mg/mL. In comparison, the reported studies by Imran proposed the use of *Syzygium cumini* leaf extract in the treatment of bacterial infections caused by drug-resistant *S. aureus* strains with MIC values ranging from 1.56 to 25 mg/mL while the ethyl acetate extract of *Syzygium cumini* leaves possessed the value of 12.5 mg/mL (Imran et al., 2017). MIC values of the *Daphne genkwa* extract and the *Magnolia officinais* extract were reported to be 14 times higher than our value (Kuok et al., 2017). The investigation by Kouk also pointed out that highest MRSA resistance activity with a MIC value of 6.25 mg/mL was disclosed in *Verbena officinalis* extract (Kuok et al., 2017). The MIC value of the *Carallia brachiata* ethyl acetate extract fraction has shown the extraordinary MRSA resistance potential as compared to previous studies.

Table 3: MIC value of ethyl acetate fractional extract

Plant names	Extract fraction	MIC (mg/mL)	
		MSSA	MRSA
Carallia brachiata L.	n-hexane	-	-
	Ethyl acetate	2,500	2,500

Cefoxitin is a new, cephalosporin-like antibiotic which is highly resistant to hydrolysis by β-lactamase. Clinical MRSA general resistance to cefoxitin antibiotics and expression of *mecA* gene. This study practice the checkerboard assay with purpose defined synergism between the fractional extracts of *Carallia brachiata L.* and cefoxitin. The results are present in Figure 2 and Table 4.

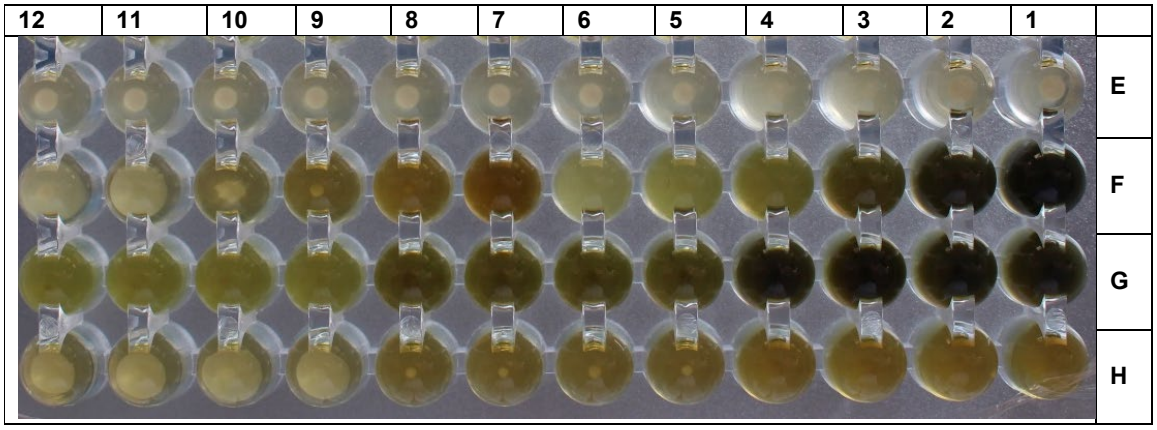

Figure 2: The checkerboard assay; H range expressed the synergism of Carallia brachiata ethylacetate fraction and cefoxitin. From well 1-well 4 of H, the results show that no growth of MRSA, from whom FIC index is calculated and showed in Table 4.

Results show that FIC index equals 1.002 (Table 4). Following the calculation formula of Kuok et al., 2017 the results indicated that *Carallia brachiata* L. ethylacetate fractional extract and cefoxitin have no synergism, which determined the target of *Carallia brachiata* L. ethylacetate fractional extract is different with the target of cefoxitin in anti-MRSA activity.

Table 4: FIC value of both fractional extracts and cefoxitin

Names	Extract fraction	MIC (mg/mL)		
		Alone	Synergism	FICI
Carallia brachiata L.	n-hexane	-	-	-
	Ethyl acetate	2,500	2,500	1.002

3.2 Hemolytic toxin inhibition capacity of *Carallia brachiata* L. extracts.

The MRSA strain is capable of producing hemolytic toxins, the primary toxin for which scientists seek inhibitors. Bacterial inhibitors of hemolytic toxins are of interest because they are generally designed to act on bacterial toxins without killing bacteria, thereby reducing selective pressure and limiting resistance phenotypes. The inhibition of hemolytic toxins by extracts of *Carallia brachiate* L. was therefore investigated. The results revealed that the ethyl acetate fraction of the Carallia brachiata extract inhibits the hemolytic toxicity of the MRSA strain. In details, MRSA was treated with n-hexane and ethyl acetate fractions of the Carallia brachiata extract. After 24 h of culturing, the bacterial culture medium was spread on the agar plates, and the plates were incubated at 36 ºC with 5 % CO_2 in the cell culture incubator. Results were recorded after 24 h of incubation. Non-treated MRSA with ethyl acetate extract fraction displayed visible hemolytic rings, whereas treated MRSA expressed bacterial growth without the detection of hemolytic rings (Figure 3). At a concentration of 4 mg/mL, preliminary results show that the Carallia brachiata extract's ethyl acetate fraction inhibits the hemolytic toxicity of MRSA.

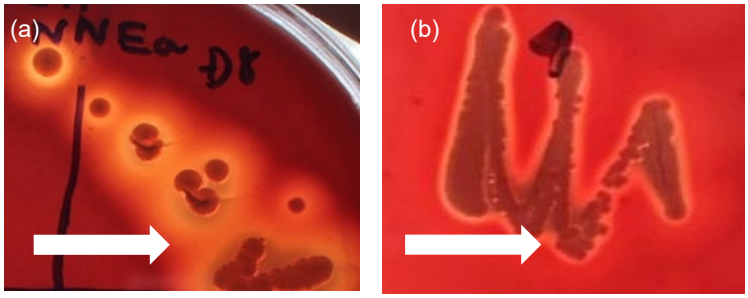

Figure 3: Hemolysis inhibition by ethyl acetate Carallia brachiata extract fraction (a) MRSA not processed with ethyl acetate Carallia brachiata extract fraction (control), (b) MRSA processed with ethyl acetate Carallia brachiata extract fraction

4. Conclusion

In this study, anti-MRSA activity of ethyl acetate fraction of the *Carallia brachiata* extract was first reported with a MIC value of 2.5 mg/L. Based on the results of the study, inhibition of the hemolytic toxin of the MRSA strain ATCC33591 was initially determined to be the effect of ethyl acetate extracts of *Carallia brachiata*. Previous studies have primarily focused on the anti-inflammatory and wound-healing properties of *Carallia brachiata* extract. The results of this study demonstrate a different aspect of the bioactivity of *Carallia brachiata* extracts.

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