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The Potency of the *Cratoxylum Cochinchinensis* L. Fractional Extract against Some Clinical Methicillin-Resistant *Staphylococcus Aureus* (MRSA) in Viet Nam

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important global priority for researching and developing new treatments. Because it takes so much time and money to discover a new antibiotic, the process is becoming more and more challenging. Faced with the above situations, finding new sources of antibiotics or new methods to control antibiotic-resistant bacteria, especially super-resistant varieties, is extremely urgent. For a very long time, medicinal herbs have been utilized to treat infectious disorders. In Vietnam, medicinal plants have been used in treating acute and chronic diseases since ancient times according to traditional medicine prescriptions. Thus, the purpose of this research is to collect the plant extract from medicinal herbs in Vietnam and then the anti-clinical MRSA of the plant extract is determined by the diffusion method. The maceration method is used for collecting the plant extracts. The power of anti-MRSA of plant extracts is determined by the microdilution method. The results show that MIC values of *Cratoxylum cochinchinense* L. fractional extract against six clinical MRSA in Vietnam are equal to 1.625 mg/mL and 3.250 mg/mL. In addition, the *Cratoxylum cochinchinense* L. fractional extract expresses the potential of anti-biofilm activity on MRSA ATCC33591.

1. Introduction

Staphylococcus aureus, one of the main culprits behind hospital and community-acquired infections, can have detrimental effects. Methicillin-resistant *Staphylococcus aureus* (MRSA) resistance to high multi-antibiotics. However, MRSA is still sensitive to vancomycin. MRSA increases the risk of multidrug resistance in Viet Nam (Bình and Hùng, 2023). Vancomycin and linezolid are first-line treatment options for MRSA infectious diseases (Vũ et al., 2023). Vancomycin is an essential antibiotic in the treatment of severe gram-positive infections in children today, especially in the context of resistance medicine is on the rise in Viet Nam (Tuấn, 2024). However, Vancomycin is at risk of harmful effects, especially nephrotoxicity, when using high doses of vancomycin. The *Cratoxylum* genus was originally used as a traditional medicinal plant in Asia. The biological activity and phytochemical analysis studies of the *Cratoxylum* species showed a variety of phytoconstituents, such as flavonoids, xanthones, triterpenoids, and phenolic compounds, which are associated with several significant pharmacological effects including anti-inflammatory, anti-cancer, antimicrobial, antifungal, antioxidant, and anti-gastric ulcer (Sánchez-Quesada et al., 2013). These findings show the based knowledge of the *Cratoxylum* genus, which is a traditional medicinal plant that is going to be researched and applied for selecting therapeutic medications for the treatment of diverse diseases (Bok et al., 2023). Therefore, the antibacterial potential of Vietnamese ethnomedicinal plants against MRSA of *C. cochinchinense* extract, which is collected in Vietnam, is noticed for study.

2. Material and methods

MRSA ATCC 33591 provided by Microbiologics, USA. Clinical MRSA provided by The Center for Bioscience and Biotechnology. The strains were cultivated in Tryton Soy Broth and Tryton Soy Agar (Neogen, USA), which were incubated in an incubator (DaiHan, Korea) at 37 °C for 18 to 24 h Fresh biomass of Nganh Nganh

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Nam (*Cratoxylum cochinchinense* L.) (leaves and young branches) were collected in Thu Dau Mot City, Binh Duong Province, between February and May.

2.2 Collection of crude ethanol *Cratoxylum cochinchinensis* L. Extract and the *Cratoxylum cochinchinensis* L. fractional extract

Following the morning collection of plant samples, the samples were cleaned twice: once with distilled water and once with tap water to get rid of any debris. *Cratoxylum cochinchinensis* L. were naturally dried in the lab at roughly 30 ºC ± 2 ºC. Following the morning collection of plant samples, the samples were cleaned twice: once with distilled water and once with tap water to get rid of any debris. *Cratoxylum cochinchinensis* L. were naturally dried at roughly 30 ºC ± 2 ºC. *Cratoxylum cochinchinensis* L. specimens were allowed to dry completely before being pureed in a mass mixer (after two or three days of weighing, the sample volume remained constant). The medicinal powder was soaked in 99.5 % ethanol for 24 h at 50 ºC (one gram of powder to ten milliliters of ethanol) to collect the crude extract. The *C. cochinchinensis* L. extract was filtered through the Whatman filter paper to remove residuals, solution of *C. cochinchinensis* L. extract was collected. A rotary evaporator was used to dry the filtrate, which resulted in a temperature of 30 °C. The ethanol *C. cochinchinensis* L. extract was centrifuged and then dried at 50 ºC until a consistent mass was achieved. For later use, this extract was kept in a refrigerator at 4 ºC. (Ingle et al., 2017). Next, a 1:1 volume ratio of methanol and water was used to dissolve the crude ethanol extract of *C. cochinchinensis*. An ultrasonic whisk was used to dissolve the extract fully in under three minutes. The mixture was then treated via liquid-liquid phase separation using n-hexane and ethyl acetate following on 1:1 (v/v) ratio between sample and solvent. The n-hexane fraction was collected by first separating with n-hexane solvent, continuously Na_2SO_4 is used for isolated H_2O to come out of the n-hexane fraction. After separating the n-ethyl acetate fraction was collected by second separating with ethyl acetate solvent, and continuously Na_2SO_4 used for isolated H_2O to come out of the n-hexane fraction. *C. cochinchinensis* fractional extract including n-hexane fractional extract and ethyl acetate fractional extract, were dried through a rotary evaporator at 30 ºC to eliminate all solvents. The original solutions, which were extract fractions in 100% DMSO, were refrigerated at 4 ºC until they were needed. For screening the antibacterial activity, the original *C. cochinchinensis* fractional solutions were diluted in 20 % DMSO for the studies.

2.3 Determination of anti-clinical MRSA activity of the *Cratoxylum cochinchinensis* L. fractional extract by dilution method

The lowest concentration at which bacterial growth is noticeably inhibited is known as the minimum inhibitory concentration or MIC (Bộ Y Tế, 2015). The MIC value of *C. cochinchinensis* L. extract against MRSA was determined through the microdilution method. Each well in the 96-well plates was supplemented with the following elements: 100 µL of *C. cochinchinensis* fractional extracts, 90 µL medium liquid (MH liquid), and 10 µL of MRSA's concentration equal to 10^6 CFU/mL. Each well had a final volume of 200 µL. A 96-well plate was used for the two-fold serial dilutions utilizing the fractions of *C. cochinchinensis* L. extract. The experiment setup was used to set up the wells, which are shown in the results section. The control group consisted of wells where only MHB was contained in the well, MHB was added MRSA in the well, MHB added DMSO in the well, and MHB was added vancomycin in the well. The 96-wells plate were cultured at 37 ºC for 24 h, aerobically. Following the incubation time, the control wells that showed bacterial growth were identified in the wells that contained MHB and MRSA, or MHB and DMSO 20 % and MRSA. The well from which the extract concentration in the series of dilutions was lowest was identified as having the minimum inhibitory concentration (MIC) value, at which there was no bacterial growth in the well. 150 µL mixture in the well were spread on three Mueller-Hinton agar plate, which continuously incubated in incubator at 37 ºC for 24 h. After 24 h, the results were recorded following MRSA growth; if MRSA was absent; or the density of MRSA cells on MHA plates was less than 300 CFU/mL, The MIC value of *C. cochinchinensis* fractional extracts was determined (Wayne, 2010), was repeated 3 times (Thanh et al., 2020).

2.4 Determination of biofilm inhibition capacity of the *Cratoxylum cochinchinensis* L. fractional Extract

Determine the ability of biofilm inhibition of the *C. cochinchinensis* L. fractional extract based on the ability to stain the dye on strain MRSA according to the method of Mataraci and Dosler (2012) (Sharafi et al., 2024) with a slight modification. Each Eppendorf tube has a final volume of 200 µl of contents including 100 µl of fraction, 10 µl of a suspension of MRSA activated overnight in the previous 24 h, at 37 ºC in a refrigerator Microbiological culture, 90 µl TSB medium + 1% glucose. Then continue incubation for 24 h at 35 °C. The medium was removed and the tubes were washed three times with 250 µl of distilled water to remove bacteria and allowed to dry. Once dry, 200 µl of solution 99 % methanol was added to each tube for 20 min for fixation. Next, the solvent is removed and the tubes are let dry for 15 min. Finally, the tubes were stained with 0.1 % crystal violet for 15 min. Use distilled water to wash off excess stains, and then these tubes are left to air dry. Elute by adding 200 µl of

clear 95 % ethanol for 10 min. The results were recorded based on the ability to stain the dye for determine of biofilm inhibition capacity of the *Cratoxylum cochinchinensis* L. fractional extract. Experiments were repeated three times.

2.5 Scanning electron microscopy (SEM)

Each petri dish contains an additional sterile 4x4 cm^2 diameter glass plate The solution is supplied which is *C. cochinchinensis* L. ethyl acetate fractional extract; 10 µl of bacterial solution grown overnight to a density of bacterial cells in the plate equal to 10^5 CFU/mL, add TSB medium with 1 % glucose to The final volume in the dish is 10 ml. Control Petri dishes with sterile glass plates contain only TSB medium and 1% glucose with bacterial suspension; The second control is the lips TSB field has 1 % glucose supplemented with cefoxitin antibiotic at a concentration equal to 128 µg/ml and the solution bacteria. Continue incubation in the incubator at 35 ºC, within 24 h. Next, the plates and slides are prepared to stain with crystal violet according to the Mataraci and Dosler 2012 method, which had been presented in the biofilm formation inhibition test. Finally, the glass slides are glued on the carbon board and inserted into the sprayer Platinum (Pt) coating. The samples are then put into the imaging chamber and photographed SEM using FESEM S4800 (Hitachi, Japan).
The assay was repeated 3 times, and the results were presented as mean ± standard deviations. STATGRAPHICS Centurion XV used for the results statistical process (Stat point Technologies, USA) (Statgraphics Centurion, 2006).

3. Results and discussion

3.1 Determination of anti-clinical MRSA activity of the *Cratoxylum cochinchinensis* L. fractional extract by dilution method

Based on Table 1. The results of determining the *Cratoxylum cochinchinensis* L. fractional extract showed activity against clinical MRSA strains in Vietnam at different concentrations depending on each strain, the high concentration of the *Cratoxylum cochinchinensis* L. fractional extract showed Anti-MRSA activity ranging from 1.625 mg/mL to 3.250 mg/mL.

Table 1. The anti-clinical MRSA activity of the Cratoxylum cochinchinensis L. fractional extract

Bacteria	TSB bacteria	+DMSO 20 %	DMSO 10 %	Vancomycin (8 mg/L)	Vancomycin (4 mg/L)	NNEa 1	NNEa 2	NNEa 3	NNEa 4	NNEa 5	NNEa 6
MSSA® 6538	+	+	+	-	-	-	-	+	+	+	+
MRSA® 33591	+	+	+	-	-	-	-	+	+	+	+
S.aureus 138	+	+	+	-	-	-	-	+	+	+	+
S. aureus 157	+	+	+	-	+	-	-	+	+	+	+
S. aureus 161	+	+	+	+	+	-	-	+	+	+	+
S. aureus 248	+	+	+	-	-	-	+	+	+	+	+

Note: NNEa: the *Cratoxylum cochinchinensis* L. fractional extract at a concentration equal to 3.250 mg/mL-0.102 mg/mL
This is consistent with the author's previously published results on the resistance to MRSA ATCC33591 of Ngang Nam ethyl acetate extract with the MIC value determined at 1.625 mg/mL.

3.2 The potential of *Cratoxylum cochinchinensis* ethyl acetate extract inhibits the biofilm formation on MRSA ATCC33591

The ability of Cratoxylum cochinchinensis ethyl acetate extract to inhibit biofilm formation on MRSA strains using the color method with crystal violet. The results are presented in Figure 1, Comparison with the control tube is MRSA cultured in TSB medium supplemented with cefoxitin antibiotic at a concentration of 8 µg/mL with crystal purple color (tube 4), two tubes containing MRSA grown in TSB medium supplemented DMSO 20 %, DMSO 10 % still stain blue. Because MRSA is resistant to the antibiotic cefoxitin, MRSA grows normally and stains darker than the tube containing bacteria with the medium. When encountering an unfavorable environment such as drugs, disinfectants, stress, etc., The biofilm formation process will be activated to create a biofilm on MRSA because the biofilm contributes to hindering drug penetration (de la Fuente-Núñez et al., 2013; Stewart & Costerton, 2001).
In Figure 1, the Eppendorfs are marked to number ranger from (1) to (7); (1) Control (TSB +MRSA); (2): control (TSB+ DMSO 20 %+MRSA); (3): control (TSB+DMSO 10 %+MRSA); (4): control (TSB+ cefoxitin 8 mg/L+MRSA); (5): TSB+MRSA + *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract at the concentration 1.625 mg/mL, (6): TSB+MRSA + *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract at the concentration 0.813 mg/mL, (7): TSB+MRSA + *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract at the concentration 0.406 mg/mL.

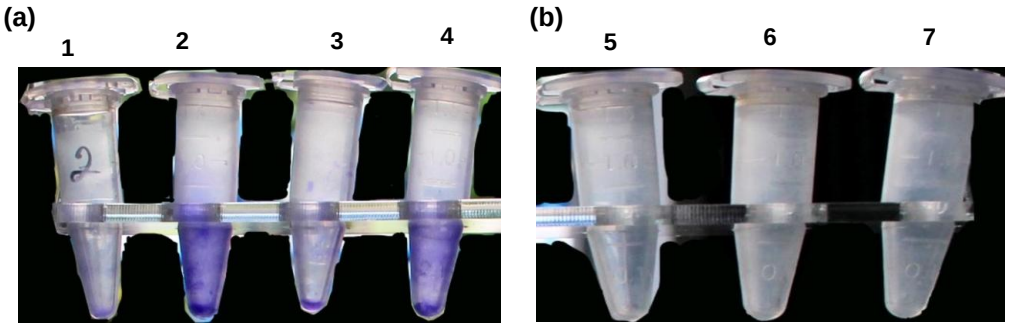

Figure 1: The Biofilms of (a) MRSA were cultured in the TSB medium which is not supplied Cratoxylum cochinchinensis L. ethyl acetate fraction extract; (b)The Biofilms of MRSA were cultured in the TSB medium which is supplied Cratoxylum cochinchinensis L. ethyl acetate fractional extract

Denpending on Figure 1b. Results determined that the concentration of the C. cochinchinensis L. ethyl acetate fractional extract inhibited biofilm formation at a concentration equal to 0.406 mg/mL. For species belonging to the genus Cratoxylum, there have been no reports on the inhibitory activity of biofilm formation on MRSA. The C. cochinchinensis L. ethyl acetate fractional extract can inhibit biofilm formation and has anti-MRSA activity, so it was chosen to conduct repeated experiments on inhibiting biofilm formation on MRSA for SEM imaging.
This result of biofilm formation inhibition activity of the C. cochinchinensis L. ethyl acetate fractional extract was first reported on MRSA. This result is compared to the study of author Arunachalam Kannappan et al, the dichloromethane extract of Vetiveria zizanioides root has been determined to have MRSA ATCC 33591 inhibitory activity of 1.024 mg/ml and biofilm formation inhibition concentration of 0.1-0.4 mg/ml (Kannappan et al., 2017). This experiment aimed to observe the morphology of MRSA bacterial cells in biofilms after treatment with ethyl acetate extract.
The results of SEM imaging of MRSA biofilms are presented in Figure 2, Figure 3, and Figure 4. In Figures 2 and 3, the control samples are biofilms in which the fractionation extract did not treat MRSA. Figure 2. shows that MRSA grown in a TSB medium supplemented with 1% glucose. Biofilms from control samples had uniform cell density and clear, round, and regular cell morphology.

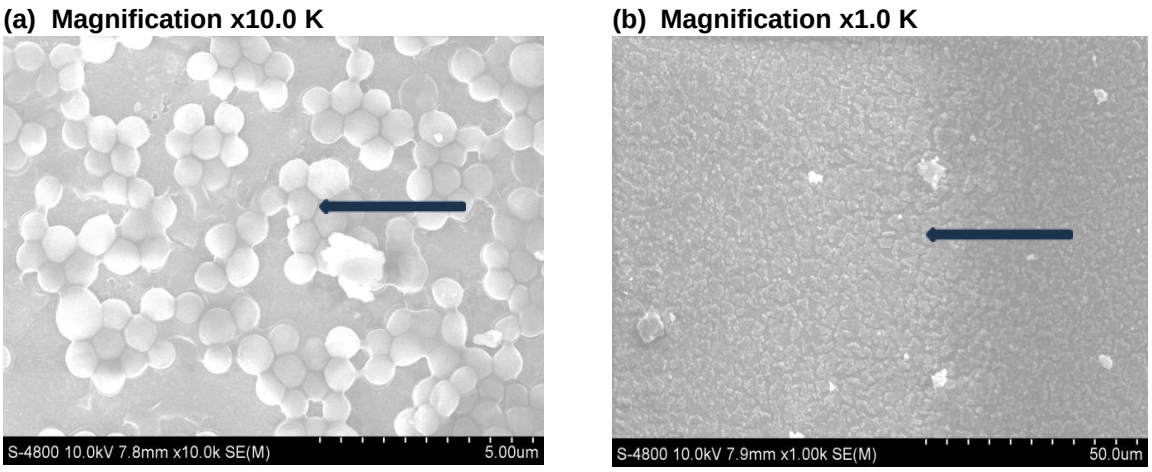

Figure 2. The biofilm formation by MRSA ATCC33591 which is cultured in the medium supplied with 1 % glucose at a) 10.0 K magnification and b) 1.0 K magnification

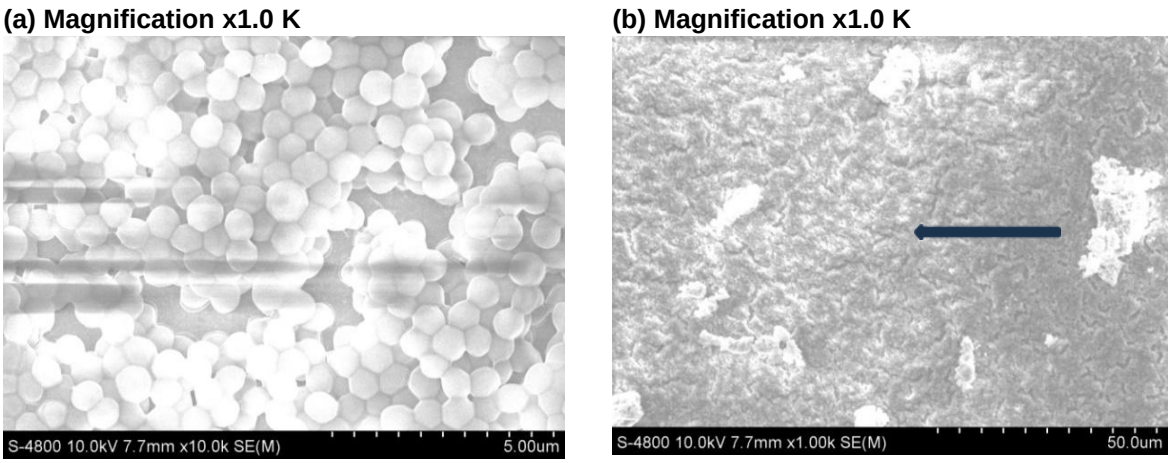

Figure 3. The biofilm formation by MRSA ATCC33591 which is cultured in the medium supplied with 1 % glucose and cefoxitin 128 mg/L) at a) 10.0 K magnification and b) 1.0 K magnification

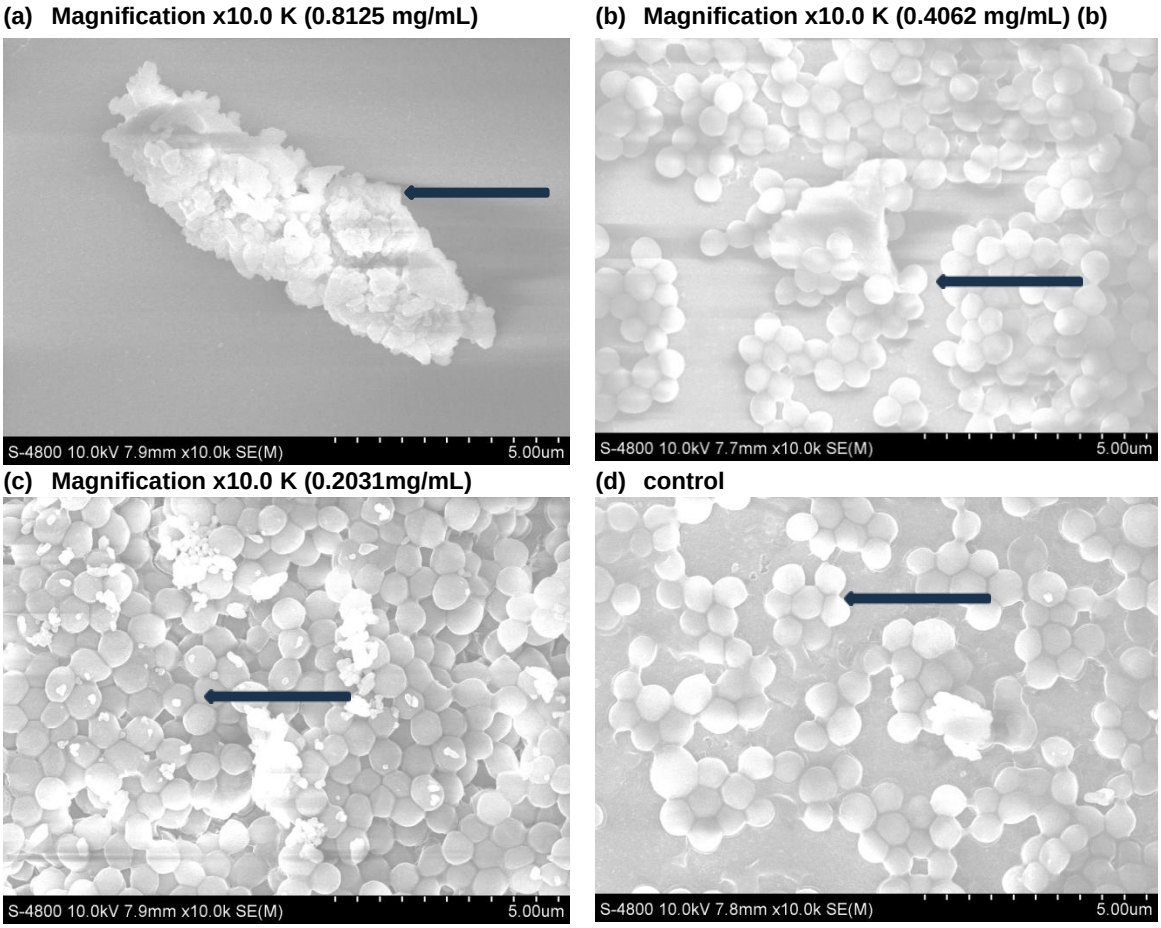

Figure 4. The biofilm formation by MRSA ATCC33591 which is cultured in the medium supplied with 1 % glucose and at (a) the concentration of the C. cochinchinensis L. ethyl acetate fractional extract equal 0.8125 mg/mL; (b) the concentration of the C. cochinchinensis L. ethyl acetate fractional extract equal 0.4062 mg/mL; (c) the concentration of the C. cochinchinensis L. ethyl acetate fractional extract equal 0.2031 mg/mL.

In Figure 3. MRSA grown in TSB medium supplemented with 1 % glucose, and the antibiotic cefoxitin at 128 µg/ml concentration. Biofilms from control samples had uniform cell density and clear, round, and regular cell

morphology. On the contrary, when TSB medium supplied 1 % glucose and the *C. cochinchinensis* L. ethyl acetate fractional extract at concentrations of 0.8125 mg/mL, 0.4062 mg/mL, and 0.2031 mg/mL, respectively, the MRSA cell morphology in the biofilm was different compared to the control.

The results are presented in Figure 4, the concentration of the Cratoxylum cochinchinensis L. ethyl acetate fractional extract is 0.8125 mg/mL, and the cell density in the biofilm decreases. Based on the SEM image (10.0kV 7.9mm x10.0K), MRSA morphology changed, and the MRSA cell wall was damaged and clustered. In the Cratoxylum cochinchinensis L. ethyl acetate fractional extract at a concentration of 0.4062 mg/mL, the MRSA cell morphology changed and clustered, but the cell density was more uniform. In the Cratoxylum cochinchinensis L. ethyl acetate fractional extract at a concentration of 0.2031 mg/mL, uniform cell density, and clear cell morphology similar to the control.

Therefore, the results indicate that the *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract has inhibitory activity on biofilm formation, and the cell wall may be the target of this fractional extract.

4. Conclusion

The research determines the clinical MRSA resistance of the *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract and the ability to inhibit biofilm formation on MRSA ATCC33591. The *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract against six clinical MRSA in Vietnam at a concentration ranging from 1.625 mg/mL to 3.250 mg/mL. The results show that the target of the impact of the *Cratoxylum cochinchinensis* L. ethyl acetate fractional extract is the cell wall of MRSA through SEM imaging.

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