

# Anti-MRSA activity of *Stereospermum fimbriatum*'s stem bark extracted using subcritical and supercritical carbon dioxide

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## Abstract

Antibiotic resistance is a major challenge in healthcare, and this is further worsened by the presence of the dreadful Methicillin-resistant *Staphylococcus aureus* (MRSA) infection. This has urged scientists to find new effective antimicrobial drugs. Earth is enriched with natural resources such as plants that have been used traditionally to cure diseases. *Stereospermum fimbriatum* or "Chicha" had been used traditionally to treat several illnesses such as stomachache, earache, itchy skin, and postpartum illness. Thus, this study was designed to investigate the antibacterial potential of *S. fimbriatum*'s stem bark against MRSA. Subcritical (Sub-CO<sub>2</sub>) and supercritical carbon dioxide (Sup-CO<sub>2</sub>) extractions were used to extract the stem bark, with and without the addition of co-solvent (ethanol). The antimicrobial assay was carried out using disc diffusion (200, 400 and 600 µg/disc), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. There was no anti-MRSA activity observed on both the Sub-CO<sub>2</sub> and Sup-CO<sub>2</sub> extracts without 10% ethanol. The most potent anti-MRSA was observed by the Sup-CO<sub>2</sub> extract with 10% ethanol with the MIC value of 500 µg/mL. Therefore, the use of ethanol (10 %) in the extraction increased the antibacterial activity of the *S. fimbriatum*'s extract against MRSA. These research findings could potentially facilitate future investigations into the utilization of green extraction methods to uncover promising antibacterial agents that can effectively target MRSA, a formidable pathogen. Future studies on the other parts of *S. fimbriatum*, its potential toxicity, and the possible mechanisms of action are needed to investigate its promising therapeutic values on MRSA infections.

**Keywords:** anti-MRSA, chicha, *Stereospermum fimbriatum*, subcritical CO<sub>2</sub> extraction, supercritical CO<sub>2</sub> extraction

## Introduction

The use of solvent extraction in herbal or medicine production has become a global concern regarding its effect on human health and the environment as well (Gil-Ch'avez *et al.*, 2012; Opuni *et al.*, 2021). Besides, the search for new effective medicines to treat infectious diseases has also increased in demand as microorganisms are getting more resistant to the available antibiotics. The

mortality rates caused by MRSA infection are considerably higher in comparison to infections caused by Methicillin-sensitive *S. aureus* (MSSA). This disparity is exacerbated by the emergence of antibiotic resistance (Garoy *et al.*, 2019). Plant-derived drugs in the market such as topotecan, vincristine, and Taxol (anti-cancer agent) are well-known for their effectiveness (Cragg and Pezzuto, 2016). Within the period of between 1981 and 2019, 1,394 small

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molecules were approved of which 33.6 % can be traced back to natural products (Newman and Cragg, 2019).

*S. fimbriatum*, commonly known as 'Chicha' or 'lempoyan' locally, earns the nickname 'snake tree' due to its elongated, coiled fruits. This species primarily thrives in lowland and hill forests and widely grows across Peninsular Malaysia, specifically in Kelantan, Terengganu, Kedah, Malacca, Langkawi, and Tioman Island. Apart from that, it is also distributed in Sumatra, Myanmar, and Laos. According to local Malaysians, the dried flowers of this plant were incorporated into desserts to impart flavour (Awang *et al.*, 2016). This plant was traditionally used for medicinal purposes, with the roots and shoots being boiled to relieve postpartum ailments and stomachaches, respectively. Additionally, the leaves were crushed to extract their juice, which was applied to treat earaches, and when combined with lime, it served as a remedy for itchy skin (Awang *et al.*, 2016). Despite being a rare plant species, it is crucial not to overlook its valuable traditional attributes, but rather delve into its unexplored therapeutic potential such as its antimicrobial properties.

Modern approaches using green technology extraction methods had been applied in drug discovery from natural resources such as subcritical and supercritical fluid extraction. Green technology extraction methods refer to environmentally friendly and sustainable techniques that minimize the use of hazardous chemicals and energy consumption while maximizing efficiency and yield. These methods align with the principles of green chemistry and are considered more eco-friendly alternatives to traditional extraction techniques. Subcritical and supercritical fluid extraction are two specific methods that have shown promise in drug discovery (Carpentieri *et al.*, 2021). Previous studies on *S. fimbriatum* had been reported using supercritical carbon dioxide and Soxhlet extractions which analysed its bioactive compounds and enriched the isolated compounds (Fadhlina *et al.*, 2020; Fadhlina *et al.*, 2021; Izyani Awang *et al.*, 2020). In view of the scanty information on *S. fimbriatum*, the present study aims at

evaluating the antibacterial activity of *S. fimbriatum* Sup-CO<sub>2</sub> and Sub-CO<sub>2</sub> extracts against *Methicillin-resistant Staphylococcus aureus* (MRSA).

## Materials and Methods

### Sample collection

The plant material (stem bark) was collected at Kampung Chicha Tinggi in Kelantan, Malaysia. It was verified by a botanist (Dr. Shamsul Khamis, Universiti Putra Malaysia) and a voucher specimen (PIIUM 0249) was deposited at herbarium, Kulliyyah of Pharmacy, International Islamic University Malaysia. The collected plant part was further cleaned and dried in a drying room for a week. After the drying process, the plant was ground into powder for extraction purposes.

### Subcritical carbon dioxide (Sub-CO<sub>2</sub>) extraction

About 200 g of dried and ground stem bark was placed in a 1 L extraction vessel of the Sub-CO<sub>2</sub> system (Figure 1A). The preliminary Sub-CO<sub>2</sub> extraction with and without ethanol (10 %) as co-solvent was performed at 28°C under the pressure of 70 bar for four hours. The extracts were collected after cycles of extraction completed with every cycle spanned for 3 to 5 minutes (Alam *et al.*, 2017).

### Supercritical carbon dioxide (Sup-CO<sub>2</sub>) extraction

Waters SFE 1000 was utilized as the Sup-CO<sub>2</sub> extraction system with a maximum working pressure of 1000 bar and a temperature range of up to 80°C, featuring a 1 L extraction chamber (Figure 1B). The extraction process was conducted according to Fadhlina *et al.* (2020), with some modifications such as, the pressure was set at 200 bar, the addition of 10% ethanol as a co-solvent ethanol, and the temperature was set at 60°C. About 20 g of powdered stem bark was loaded into the extraction chamber. The CO<sub>2</sub> was supplied from a gas cylinder and compressed using a diaphragm compressor to the desired

pressure, which was then regulated by the pressure controller and heated to the desired temperature via a heat exchanger to achieve the supercritical state. The CO<sub>2</sub>, along with the extracted material, was

depressurized at the extractor exit to separate the material. The flow rate was kept constant at 2g/min and the average yield of three experiments was recorded.

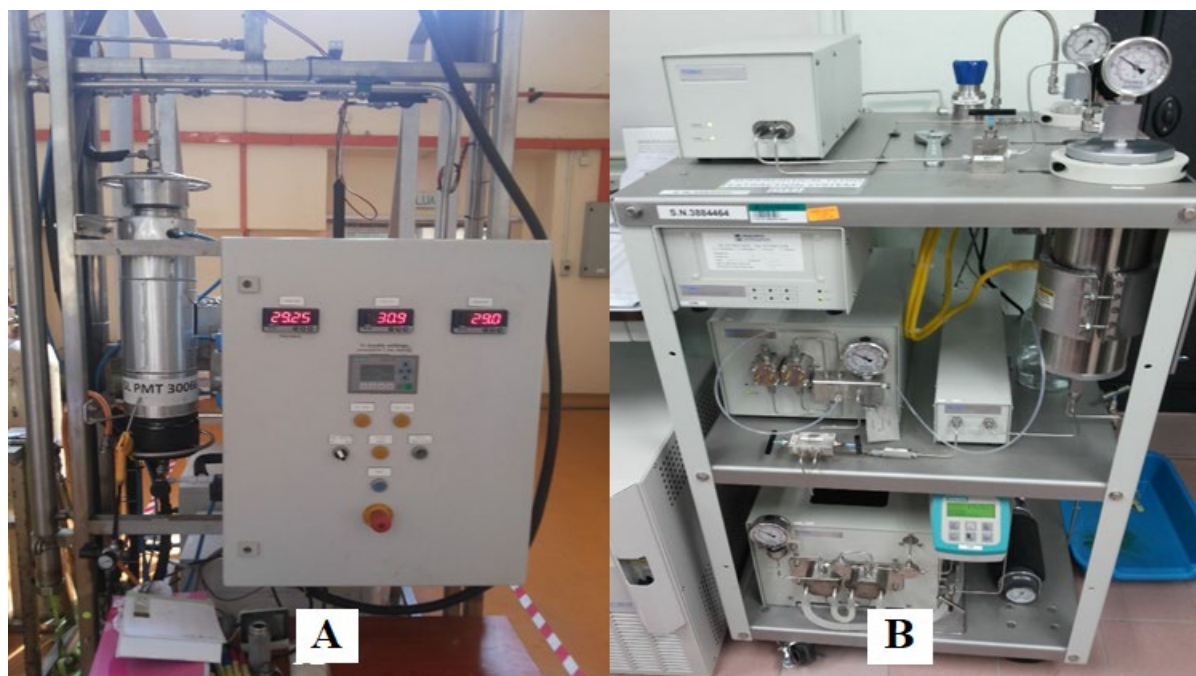


Figure 1. Subcritical (A) and Supercritical (B) CO<sub>2</sub> extractor.

## Sample preparation

The sample was prepared by dissolving 10 mg of the sample in 1 mL of dimethyl sulfoxide (DMSO). This stock sample was stored in the chiller (4°C) until further use.

## Microorganisms

A clinically isolated Methicillin-resistant *S. aureus* (MRSA) was used in the antimicrobial assay. The inoculum preparation was done using 0.5 McFarland standard which provided the standard optical density (OD) of MRSA. Single colonies growth on agar culture was transferred into growth medium, Mueller-Hinton Broth (MHB). Incubation took place at 37 °C for 24 hours. A spectrophotometer was used to check the OD of the incubated inoculum at 600 nm (Fadhline *et al.*, 2021).

## Antimicrobial assay

### Disc diffusion method

A disc diffusion assay was carried out to screen for the anti-MRSA activity of each sample. All extracts were tested in three different dosages which were 200, 400, and 600 µg/disc. For disc preparation, filter paper number three was punched and sterilized in the autoclave. Using sterile forceps, each of the extracts was loaded into the discs and dried for 30 minutes. The inoculum adjusted to 0.5 McFarland standard was swabbed (100 µL) on the Mueller-Hinton agar plate evenly. The loaded discs were laid on the inoculated agar and incubated. The positive control used for this test was vancomycin (30 µg/disc), while for the negative control, the solvent carrier, DMSO was employed. All tests were done in triplicate and the mean from triplicate measurements of the inhibition zone

(diameter) with standard deviation was recorded (Fadhlina *et al.*, 2021).

### Micro-dilution method

The MIC of extracts was determined by the broth micro-dilution test according to the previous method (Fadhlina *et al.*, 2021) with some modifications. Adjusted inoculum using 0.5 McFarland standard was loaded into 96-well plates and 3  $\mu$ L of the sample stock (10 mg/mL) was transferred into the inoculated well (197  $\mu$ L). Then, different concentration of the sample was prepared (1000-7.8  $\mu$ g/mL) with the final volume of 100  $\mu$ L each well. The tested plate was incubated at 37 °C for 24 hours. The positive control (antibiotic) was tested in the same manner as the stated steps (100-0.2  $\mu$ g/mL). After the incubation period, an indicator which was 0.01% (wt/v) resazurin sodium salt (30  $\mu$ L) was added to each well and incubated for two hours. MIC was considered as the lowest concentration that preserved the blue or purple colour of resazurin. Treated cultures with MIC value and above were swabbed onto the agar plate. MBC was considered as the concentration that give no growth upon completion of the incubation period.

### Statistical analysis

IBM SPSS Statistics 20 was used for the statistical analysis. The results were presented as the means of three readings  $\pm$  the standard deviation (SD). Statistical significance was determined using one-way ANOVA analysis, followed by post-hoc test, with a significance value set at  $p < 0.05$ .

### Result and Discussion

Supercritical carbon dioxide (Sup-CO<sub>2</sub>) and subcritical carbon dioxide (Sub-CO<sub>2</sub>) extraction are two commonly used methods for extracting plant extracts. Both extractions are an eco-friendly process that does not require the use of harmful solvents and have their advantages and disadvantages in terms of extraction efficiency as well as biological activities (Awang *et al.*, 2016). Sup-CO<sub>2</sub> extraction

involves using CO<sub>2</sub> at temperatures and pressures above its critical point, which results in a highly efficient extraction process. This method is preferred for extracting non-polar and semi-polar compounds, such as essential oils, fatty acids, and some alkaloids. The extraction efficiency of Sup-CO<sub>2</sub> is generally higher than that of Sub-CO<sub>2</sub>, due to its ability to extract a wider range of compounds. On the other hand, subcritical carbon dioxide (Sub-CO<sub>2</sub>) extraction involves using carbon dioxide at temperatures and pressures below its critical point, which results in a less efficient extraction process compared to Sup-CO<sub>2</sub>. This method is preferred for extracting polar compounds, such as flavonoids, glycosides, and some alkaloids. While Sub-CO<sub>2</sub> extraction is less efficient, it has some advantages over Sup-CO<sub>2</sub> extraction. For example, Sub-CO<sub>2</sub> extraction can preserve the bioactivity of heat-sensitive compounds and can also extract a broader range of polar compounds (Gallego *et al.*, 2019).

The present study was the first work to report on the Sub-CO<sub>2</sub> extraction of *S. fimbriatum* using an eco-friendly co-solvent, ethanol. The percentage of yields (Table 1) for extract with the use of 10 % ethanol as co-solvent (6.32 %) was more than the percentage of yields obtained without the use of ethanol (4.19 %). The anti-MRSA activity (Table 2) of Sub-CO<sub>2</sub> extracts were only observed on the stem bark extract with the addition of 10% ethanol (Figure 2). A similar observation was recorded for Sup-CO<sub>2</sub> extract with 10% ethanol. However, there was no extract yielded by Sup-CO<sub>2</sub> extraction without the addition of 10% ethanol. All the anti-MRSA activity observed was in a dose-dependent manner (8 -10 mm). Extracts that exhibit an inhibition zone of 13 mm or greater are classified as potent extracts (Fadhlina *et al.*, 2021), and achieving this level of potency may be possible by increasing the dosage of the extract since the concentrations employed in this study were relatively low. Based on the micro-dilution assay of the active extracts (Table 3), Sup-CO<sub>2</sub> extract exhibited the lowest MIC value of 500  $\mu$ g/mL, while its MBC value was 1000  $\mu$ g/mL, against MRSA.

Table 1. The yield of Sub-CO<sub>2</sub> and Sup-CO<sub>2</sub> extraction with and without 10% ethanol.

Stem bark (200 g)	Condition	Yield (%)
Sub: CO <sub>2</sub>	70 bar at 28°C	4.19
Sub: CO <sub>2</sub> + 10 % ethanol	70 bar at 28°C	6.32
Sup: CO <sub>2</sub>	200 bar at 60°C	0.00
Sup: CO <sub>2</sub> + 10 % ethanol	200 bar at 60°C	4.15

Table 2. Disc diffusion assay of extracts against MRSA.

Extracts	CO <sub>2</sub> (mm)			CO <sub>2</sub> + 10 % Ethanol (mm)			Antibiotics (mm)
	200	400	600	200	400	600	V30
	µg	µg	µg	µg	µg	µg	
<b>Subcritical</b>	NI	NI	NI	7.67*±0.58 <sup>c</sup>	9.33±0.58 <sup>b</sup>	10.67±0.58 <sup>b</sup>	19.33±0.58 <sup>a</sup>
<b>Supercritical</b>	NI	NI	NI	8.33±0.58 <sup>c</sup>	9.67±0.58 <sup>b</sup>	10±0.00 <sup>b</sup>	

\*=Partial inhibition; NI=No inhibition; V30=Vancomycin 30 µg/disc; a, b, c: Small superscript alphabets represent post-hoc analysis tested at *p*<0.05.

Table 3. Micro-dilution assays of extracts against MRSA.

Extracts (ug/mL)	MRSA	
	MIC	MBC
<b>Sub-CO<sub>2</sub> + 10 % Ethanol</b>	>1000	-
<b>Sup-CO<sub>2</sub> + 10 % Ethanol</b>	500	1000
<b>Vancomycin</b>	0.78	1.56

Sub-CO<sub>2</sub> and Sup-CO<sub>2</sub> offer a valuable option for green technology extraction as it uses a non-toxic solvent of carbon dioxide and less solvent consumption (Awang *et al.*, 2016) with only 10 % co-solvent so that it increased the polarity and allowed the tuning of compound’s solubility in the plant extraction. The addition of 10 % ethanol at a constant temperature and pressure in both of the extraction methods might increase the solubility of antimicrobial compounds in the stem bark of *S. fimbriatum*. A similar observation was reported in a previous study on the extraction of bilberry using Sub-CO<sub>2</sub> extraction whereby it was found that the addition of 10 % ethanol as a co-solvent had

increased the efficiency of anthocyanin recovery as well as the bioactivity (Babova *et al.*, 2016). Meanwhile, a previous study on *S. fimbriatum* (Fadhlina *et al.*, 2020) using Sup-CO<sub>2</sub> extract (addition of 6% ethanol) showed lower MIC value against MRSA, operated at 40°C and 300 Bar. A novel anthraquinone compound had been isolated from the stem bark of *S. fimbriatum* which was semi-polar (Izyani Awang *et al.*, 2020). Thus, further optimization on the extraction conditions (pressure, temperature, % of co-solvent) may be conducted to enrich its bioactive compounds and improve its antimicrobial activity against a broad range of microorganisms.

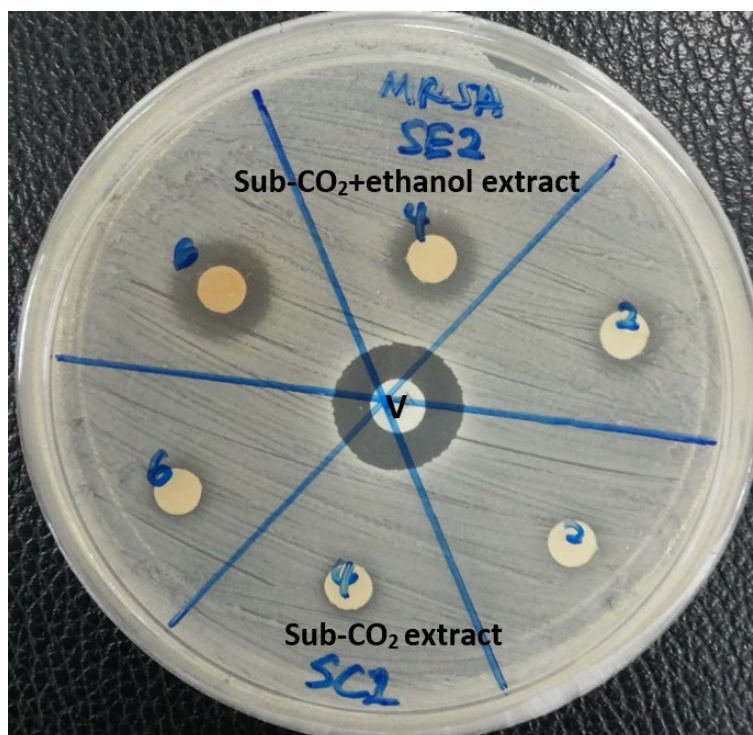


Figure 2. Inhibition zones of Sub-CO<sub>2</sub> extracts against MRSA in disc diffusion assay. 2: 200 µg/disc; 4: 400 µg/disc; 6: 600 µg/disc; V: Vancomycin 30 µg/disc.

## Conclusion

The findings of this study highlight the potential of Sup-CO<sub>2</sub> in discovering valuable antibacterial agents for targeting MRSA. Sup-CO<sub>2</sub> extraction of *S. fimbriatum*'s stem bark operated at 60°C, 200 Bar, and the addition of 10% ethanol showed higher antibacterial activity against the dreadful bacteria, MRSA, compared to Sub-CO<sub>2</sub> extraction. The use of 10% ethanol in both extractions is crucial to improve the yield and anti-MRSA activity of *S. fimbriatum* extracts. Further studies on optimizing the extraction of antibacterial extracts are needed to obtain an optimum condition against a broader range of microorganisms. The present study focused mainly on the stem bark of *S. fimbriatum*, highlighting the need for future research to explore other plant parts and assess their potential toxicity or side effects. Moreover, it is essential to further investigate the possible mechanisms responsible for the demonstrated anti-MRSA activity.

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