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The Potential anti-Methicillin Resistant *Staphylococcus Aureus* of *Cleistocalyx Operculatus* Leaf Extract and its Application in Kombucha Tea Fermentation in Vietnam

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The clinical management of methicillin-resistant *Staphylococcus aureus* (MRSA) infections is more and more challenging due to its increasing incidence. In particular, MRSA bacteria exposed to β-lactam antibiotics may exhibit genetic mutations that reduce their susceptibility to these antibiotics. The need to discover new and efficient alternatives is growing as multi-drug-resistant bacteria become more common. Plants are now recognized as natural sources of bioactive substances that have the potential to be highly effective in treating infectious diseases. Plants contain abundant secondary metabolites, several of which have demonstrated antibacterial properties in vitro. Vietnam frequently harnesses the well-known medicinal plant *Cleistocalyx operculatus* R. (*C. operculatus* R.) as an ingredient in tonic beverages. The antibacterial properties of an ethanolic extract derived from the leaves of *C. operculatus* R. are determined using the agar diffusion method. A kombucha culture, which is a symbiotic culture of bacteria and yeast, is used to aerobically ferment a sweetened tea infusion to create kombucha tea. Due to the supposed health benefits of kombucha, it has gained popularity among consumers. As the demand for high-quality kombucha beverages rises, brewers are seeking guidance from the scientific community to refine their products and meet evolving consumer expectations. Brewers are turning to the scientific community's understanding of kombucha in an attempt to better tailor their products to meet changing consumer expectations. Therefore, the use of *C. operculatus* leaves is employed in the fermentation process to produce Kombucha tea, as indicated in research on *C. operculatus* Kombucha. The anti-MRSA activity of the *C. operculatus* extract is reported with a diameter of the inhibited zone equal to 13.50 $\pm$ 0.71 mm at the concentration of 10 mg/mL, marking the first time such results have been achieved. The tea is made from *C. operculatus* leaves follows an optimal ratio between adding SCOBY and sugar tea solution is 1:3 mL/mL, and the time fermentation is 4 days, at room temperature (31.5 °C). Furthermore, the formulas of *C. operculatus* Kombucha, in synergy with sugarcane juice, are determined to have an optimal ratio of 1:3 mL/mL, preferred by approximately 40 % of customers. The results of this research contributed to the development of new Kombucha formulas, highlighting the synergy between the traditional medical plant fermented by SCOBY and the potential of industrial drink production.

1. Introduction

Septicemia, endocarditis, pneumonia, skin and soft tissue infections, bone and joint infections, and hospital-acquired infections are usually associated with MRSA. In particular, methicillin-resistant Staphylococcus aureus (MRSA) is linked to high morbidity and mortality, and remains a significant public health problem (Nazli et al., 2024). In Vietnam, MRSA is a prevalent cause of infectious diseases, among 3,709 strains of pathogenic bacteria isolated, the rate of isolated *S. aureus* was 29.9 % (Bình et al., 2023). The methicillin-resistant *Staphylococcus aureus* (MRSA) gained prominence as a superbug due to its ability to resist a majority of antibiotics and treatments used for infections. Moreover, the new antibiotic therapy for MRSA infectious is

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exclusive and long-term, which can significantly impact the quality of life for patients (Gopikrishnan and Haryini, 2024).

The Voi is a Vietnamese name for a plant distributed in Asia, known scientifically as *Cleistocalyx operculatus* R. (*C. operculatus* R.). In Vietnam, the tree grows wild and is cultivated throughout the provinces, mainly for the leaves used in making drinking water. In 1968, Nguyen Duc Minh from Department of Experimental Oriental Medicine at the Oriental Medicine Research Institute researched the antibiotic properties of the leaves and other parts of the *C. operculatus* R. tree against certain bacteria. The results indicated that leaves and buds exhibit antibiotic effects at all stages of development, with the leaves having antiseptic properties. New research by the Institute of Oriental Medicine aims to apply these properties in treating variuos intestinal diseases, sore throats, and skin ailments (Do, 2004). The leaves *C. operculatus* R. aqueous and ethanolic extracts demonstrate antibacterial activity against prevalent pathogenic strains. Research indicates that ethanolic extract has a greater impact than aqueous extract, with a minimum inhibitory concentration (MIC) of 0.4 mg/ml against *S. aureus* (Vân et al., 2021). Nonetheless, the anti-MRSA effects of *C. operculatus* R. extract has not yet been reported. In Vietnam, dried *C. operculatus* R. leaves are commonly used to make tea on a daily basis. Notably, Kombucha, a health-centric drink, is easily made and comsumed daily. Kombucha, a traditional Asian beverage, is made by fermenting tea through a symbiotic consortium of bacteria and yeasts (SCOBY) and allowing it to sit at room temperature for seven to fourteen days (Coelho et al., 2020).

The functional beverage market is one of the fastest growing segments in the world functional food market. Consumer demand for food and goods "healthy" drinking is increasing. Kombucha tea is recorded as a potential drink that helps to prevent bacterial infections, fights stress and cancer reduces haemorrhoids, actively affects cholesterol levels and facilitates excretion of toxins. Overview of the benefits of Tea Kombucha, tea fermentation, and current research, contribute to the direction of the continued research into Kombucha, aiming to diversify these beverage industries.

Given these points, the potential synergy between the anti-MRSA activity and the fermentation of *C. operculatus* R. leaves in making Kombucha tea is of interest. Additionally, the use of *C. operculatus* R. leaves for fermenting Kombucha tea have not still been reported. This study aims to investigate the anti-MRSA activity and the recipes of making Kombucha tea from *C. operculatus* R. leaves collected in Binh Duong, Vietnam.

2. Methodology of research

2.1 Materials

MRSA ATCC 33591 was supplied by Microbiologics, USA. Fresh biomass of Voi (*C. operculatus* R.) leaves were collected from May to September, located at 10.996156 " North; 106.668712 " East. The Scoby Kombucha was obtained from a Vietnamese commercial Kombucha that is kept in Thu Dau Mot University's lab

2.2 Moisture measurement

The weight ranging from 5 g to 10 g of *C. operculatus* R. leaves is pre-weighed. These leaves are then contained in a cup with a proper lid to measure moisture content. The correct weighing cup must be used to ensure that the pharmaceutical layer is no thicker than 5 mm. After opening the lid of the cup holding the medicinal herbs, place it in the drying cabinet and let it dry for one hour at 105 °C. Once the cup has cooled, move it to a dehumidifier, cover it, and weigh it. Repeat this process until there is no more than 0.5 mg difference between the two masses.

The formula uses moisture (%) to indicate the specimens' moisture content, m_1 is presented to be the mass of the fresh *C. operculatus* R. leaves (g), and m_2 is the mass dried *C. operculatus* R. leaves. The following Eq(1) was used to calculate the specimens' moisture (%) content is shown that (Mai et al., 2023):

$$\text{Moisture} = (m_1 - m_2) / m_1 \times 100\ \% \quad (1)$$

2.3 Total ash content determination

When a medicinal herb is burned completely, the inorganic residue left behind is known as total ash. This process involves using a 35-mm porcelain cup, which is heated and then cooled. The *C. operculatus* R. leaves contained in the cup are then weighed. Initially, one to five grams of chopped medicinal herbs are added to a crucible to determine their weight. Gently burning is carried out at first, gradually increasing the heat until the therapeutic plants are fully burned. Once the cup reaches a consistent mass, it is placed in an oven at 300 °C. After the herbs turn completely inorganic, they are weighed and allowed to cool in a dehumidifier.

Eq(2) was utilized to compute the aggregate ash content of the specimens. Total ash content, expressed as a percentage, is denoted by X (%) in Eq(2) (Mai et al., 2023)

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

2.4 The crude ethanol *Cleistocalyx operculatus* R. leaf extract is collected and screened the potential anti-MRSA of *Cleistocalyx operculatus* R. leaves extract

C. operculatus R. leaves are collected from 7 AM to 9 AM, then cleaned with distilled water to remove any dirt. These leaves are naturally dried in the lab at around 30 °C ± 2 °C. The specimens are further dried and processed into a powder using a mass mixer, ensuring consistency in weight after multiple weighing iterations (the sample weight doesn't change after two to three times weighing). The dried *C. operculatus* R. leaves powder was extracted using a thermostatic shaker set at 100 rpm and soaked in 99.5 % ethanol with a ratio of 1: 10 w/v for 24 h at 50 °C. Subsequently, the ethanol solution of *C. operculatus* R. leaves are filtered through Whatman filter paper to eliminate any residues. The filtrate is then dried using a rotary evaporator at 30 °C. The resulting *C. operculatus* R. leaf extract is dissolved in 100 % DMSO, forming an original solution stored at 4 °C for further procedures (Mai et al., 2020).

The efficiency of the crude *C. operculatus* R. leaves extract is determined using Eq(3). In this Equation, E is the efficiency of the crude *C. operculatus* R. leaf extract, which is presented the percentage; The molecular weight of the crude *C. operculatus* R. leaf extract is presented m (g), The molecular weight of the *C. operculatus* R. leaves (g) are presented m $_{\text{dried } \textit{Cleistocalyx operculatus} \text{ R. leaves}}$. *C. operculatus* R. leaves are dried at the room temperature (Phung, 2007).

$$\text{E}\ (\%)\frac{\text{m (extract)}}{\text{m } \textit{dried Cleistocalyx operculatus} \text{ R. leaves}}\text{x}100\ \% \quad (3)$$

For examining the antibacterial potential of the extracts, Muller-Hinton Agar (Neogen, USA) was used as the standard medium. The Kirby-Bauer method was selected to qualitative asses the anti-MRSA activity of *C. operculatus* R. leaves extract. Wells with a diameter of 4 mm were prepared on the plate. The plate is supplied with 100 µL of a MRSA suspension of 10^6 CFU/mL concentration. Well No. 1 received 100 µL of cefoxitin (at a concentration of 10 mg/mL), while Well No. 2 received 100 µL of DMSO 20 %. Well No. 3 and Well No. 4 were treated with 100 µL of the crude extract of *C. operculatus* R. leaves (10 mg/mL). The plates were then incubated for 18 h – 24 h at 37 °C, with three rounds of experiments carried out. After the incubation period, the sizes of the inhibitory zones were measured in milli-meters (mm) (Bauer et al., 1966). The results were validated using references from CLSI papers (Mai et al., 2020).

Statistical analysis was performed using STATGRAPHICS Centurion XV by Statpoint Technologies (USA) (Statgraphics Centurion, 2006).

2.5 Screening on the optimal formula for fermentation of the Kombucha tea with *Cleistocalyx operculatus* R. leaves

The fermentation of *C. operculatus* R. leaves Kombucha tea according to the manufacturer's instructions with 2 g of *C. operculatus* R. *leaves*, 400 mL of water, 75 g of sugar, specific steps are as follows: *C. operculatus* R. *leaves* are boiled with sugar and distilled water, then filtered by the Whatman filter paper, and cooled. This mixture is supplemented by SCOBY and incubated at 30-40 °C for 4 days (Ngọc et al., 2012).

The sensory evaluation method involved surveying 100 people (aged 20-22 years old, normal health). The process included mixing Kombucha leaf tea and sugarcane juice in a specific ratio. Each participant tested 9 samples sequentially and chose their favorite after 5-10 min. They then completed a sensory evaluation sheet based on criteria of color, smell, and taste, using specific symbols (111, 112, 113, 114, 115, 511, 411, 311, 211), which denote the ratios of Kombucha tea to sugarcane juice (v/v) mL/mL as 1:1, 1:2, 1:3, 1:4, 1:5, 5:1, 4:1, 3:1, and 2:1, respectively, as detailed in Table 3.

For colour criteria, judges selected one of four options by marking "X" in the "YES" box in the sensory sheet: Bright yellow with no residue (Criterion 1), Light yellow with no residue (Criterion 2), Dark yellow with low viscosity and clear residue (Criterion 3), or Dark opaque yellow with lots of residue (Criterion 4). Each judge earned "1 point" for the selected criterion and "0 points" for unselected ones.

Similarly, for smell criteria, judges chose between Light, impressive fragrance (Criterion 1), Lightly fragrant and faintly sour (Criterion 2), Little aroma with strong sourness (Criterion 3), or not fragrant but sour (Criterion 4), earning points as per their selections.

In taste criteria, judges selected criteria such as Sweet, pleasant, mildly sour with characteristic aftertaste (Criterion 1), Medium sweet and sour (Criterion 2), Strongly sweet with no aftertaste (Criterion 3), or Sour, pungent, and irritating taste (Criterion 4), with points awarded accordingly.

3. Results and discussion

3.1 Moisture measurement and Total ash content determination of *Cleistocalyx operculatus* R. leaves

C. operculatus R leaves are used to make tea every day as a traditional medical plant in Vietnam. Experiments measure the moisture and total ash content to determine the standards of *C. operculatus* R leaves as raw materials for Kombucha tea production. The results of the moisture and total ash content of *C. operculatus* R leaves are presented in Table 1. If the anti-MRSA activity of *C. operculatus* R extract is determined, *C. operculatus* R will be required to be a medical plant following the standards of medical herbs in Vietnam Pharmacopoeia Volume 2.

Table 1: Moisture and total ash content of plant specimens

Plant names	Moisture (%)	Total ash content (%)
Cleistocalyx operculatus R.	59.669 ± 0.311	± 0.516

Comparison with species in the genus *Syzygium,* the results presented in Table 1. show that the total ash content of *C. operculatus* R. leaves is higher than approximately 3 times the total ash content of *Syzygium laetum* and *Syzygium occidentale* in the study of V. R. Snehalatha and V. R. Snehalatha (Snehalatha and Rasmi, 2022). Following on criteria of medicinal herbs of Vietnam Pharmacopoeia Volume 2 in Vietnam, the total ash percentage of various herbs varies, it cannot be more than 24 % (Appendix 9.8, Vietnam Pharmacopoeia Volume 2) (Bộ, 2018), so *C. operculatus* R. leaves have been used as a medical plant.

3.2 Determination of anti-MRSA activity of the *Cleistocalyx operculatus* R. extract by the diffusion method

MRSA, an infectious disease increasingly associated with antibiotic resistance, has become a significant concern. The anti-MRSA activity of *C. operculatus* R. leaf extract has been evaluated. Additionally, the leaves of *C. operculatus* R. meet the required standards for moisture and total ash content in medicinal plants. These findings provide a favorable basis for further application and research.

The Eq(3) is used to calculate the efficiency of the crude ethanol extract of *C. operculatus* R. leaves (Phung, 2007). The results indicate an efficiency approximately three times higher (6.914 ± 0.020 %) compared to a study of Ngo Thai Bich Van et al (Vân et al., 2021).

The anti-MRSA activity of the *C. operculatus* R. extract is determined through the diffusion method, the result is presented in Figure 1.

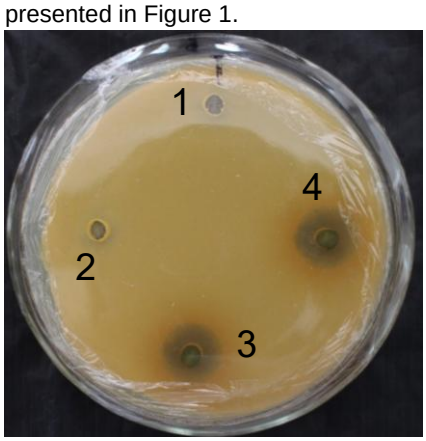

Figure 1. The potential of anti-clinical MRSA activity of the C. operculatus R. extract by the diffusion method

In Figure 1. Well No.1 contains cefoxitin at a concentration of 10 mg/mL; Well No. 2 contains DMSO 20 %; Wells No. 3 and well No. 4 contain *C. operculatus* R. extract in DMSO 20 % at a concentration of 10 mg/mL. The diameter of the inhibited zone of *C. operculatus* R. extract on MRSA ATCC33591 is 13.50 $\pm$ 0.71 mm, compared to control Well No.1 and Well No.2 DMSO 20 % (absent of the MRSA inhibited zone). The results indicate the effectiveness of *C. operculatus* R. extract against MRSA ATCC33591. The antibacterial efficacy of the extract of *C. operculatus* R. was assessed in the study of Vivek K. Bajpai et al, in which the result showed that the diameter of inhibition zones extracts (1,500 µg/disc) equivalent the concentration of *C. operculatus* R extract equals 15 mg/mL (Bajpai et al., 2010). It means that, the result of this research is suitable for the of Vivek K. Bajpai's study. In the other study, the anti-*Staphylococcus aureus* ATCC6538 activities of methanolic extract of leaves of *C. operculatus* R. grown in Vietnam is determined, in which the Inhibition zone diameter (mm) on

Staphylococcus aureus ATCC6538 equals 12 mm (Nguyen et al., 2017), is smaller than the diameter of the inhibited zone of the *C. operculatus* R. extract on MRSA ATCC33591 is 13.50 $\pm$ 0.71 mm , significance.

3.3 Screening on the optimal formula for fermentation of the Kombucha tea with *Cleistocalyx operculatus* R. leaves

3.3.1 The optimal ratio between SCOBY and the sugar-tea extract

According to Table 2, the optimal ratio for adding SCOBY and sugar tea solution is 1:3 mL/mL. After 4 days of fermentation, the product exhibits attractive qualities such as a light yellow color, slightly sour taste, and moderate sweetness.

Temperature significantly affects the fermentation process and sensory aspects of the product. Fermentation occurs faster at higher temperature (37 °C to 40 °C), but may result in poor color, smell, or taste. Fermenting the product at room temperature (31.5 °C) provides optimal sensory results. Since the SCOBY is killed at 50 ºC and inhibited at 4 ºC, extreme temperatures (too high or too low) can inhibit the fermentation process.

Time also plays a crucial role in the sensory properties of the product. The ideal fermentation period for Kombucha tea is 4 days. These conditions of fermented process are compared with the results of Silvia Alejandra Villarreal-Soto's overview about conditions of Kombucha tea fermented process, in which optimal of conditions are ranging 7 days to 15 days, temperature values ranging 37 °C to 42 °C. Temperature affects the fermentation ability as well as the sensory values of the product. The fermentation process happens faster when the temperature increases from 37 °C to 40 °C, which is suitable to the study of Vitas et al (Vitas et al., 2013). Fermenting the product at room temperature (31.5 °C) gave optimal sensory results with Light yellow, slightly sour, and moderately sweet. If the temperature is too high, the fermentation process cannot take place, because The SCOBY is killed at 50 °C. On the contrary, low temperature also prevents fermentation from taking place, because the growth of SCOBY is inhibited at 4 °C. The time factor affects the sensory properties of the product, in this study the optimal time is more 4 days, which is shorter than the results of Silvia Alejandra Villarreal-Soto's overview.

Table 2. Screening the optimal ratio between SCOBY and the sugar-tea extract

The ratio between SCOBY and the sugar-tea extract (mL/mL)	Color	fragrant smell	Taste
1:1	Light yellow	Strongly sour	Less sweet
1:3	Light yellow	Slightly sour	moderately sweet
1:5	Light yellow	Slightly sour	Less sweet
1:7	Light yellow	Slightly sour	much sweet
1:9	Light yellow	Slightly sour	much sweet

3.4 Sensory evaluation of combined Kombucha tea product sugarcane juice

The study involved 100 participants to evaluate color, smell, and taste preferences, with results shown in Table 3. The optimal ratio (v/v) between Kombucha tea and sugarcane juice is determined to be 1:3 mL/mL, which is preferred by most people for its slightly sweeter and more sour taste.

Table 3. Sensory evaluation of combined Kombucha tea product sugarcane juice

The ratio between Kombucha tea and sugarcane juice (mL/mL)	Percentage of samples selected according to color criteria (%)	Percentage of samples selected according to smell criteria	Percentage of samples selected according to taste criteria
1:1	8	15	8
1:2	0	0	8
1:3	23	38	31
1:4	38	15	15
1:5	23	0	0
5:1	0	15	23
4:1	8	15	15
3:1	0	0	0
2:1	0	0	0

Consumers favor Kombucha products with a bright yellow color, no residue, and a slightly sweet and sour taste profile. Characteristics like an impressive appearance, pleasant fragrance, and medium level of sweetness and sourness are also preferred.

This result determined that the optimal ratio (v/v) between Kombucha tea and sugarcane juice equal 1:3 mL/mL. The ratio of 1:3 mL/mL has the highest number of people choosing it, the ratio has 3 times more sugarcane juice added than Kombucha and is the most loved by many people, with a slightly sweeter and more sour taste. Bright yellow, no residue, characteristic of the product are criteria fermented the most favorite, from that determined the trend of consumers for the Kombucha products. In addition, light impressive, pleasant fragrances and Medium sweet and sour, not harsh for taste criteria are the favorite of consumers. The synergy between *C. operculatus* R. Kombucha tea and sugarcane juice is first reported, this product is chosen because of a slightly sweeter and more sour taste, the trend of consumers for the Kombucha products. In addition, light impressive, pleasant fragrances and Medium sweet and sour, not harsh for taste criteria are the favorite of consumers.

4. Conclusion

The research evaluates the anti-MRSA activity of *C. operculatus* R. leaves extract and determines optimal fermentation conditions using a 1:3 mL/mL ratio of SCOBY and sugar tea solution, 4 days of fermentation, at room temperature 31.5 °C. The combination of Kombucha tea and sugarcane juice at a 1:3 mL/mL ratio is found to be the optimal choice for consumers. Diversify products from *C. operculatus* R. leaves to increase the value of traditional medical materials. The optimal conditions of *C. operculatus* R. leaf fermented Kombucha tea are undertaken to be assessed to save time and money to produce the Kombucha tea.

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