IN-VITRO ANTIBACTERIAL ACTIVITY OF THE FRACTIONS FROM *CLEISTOCALYX OPERCULATUS* (ROXB.) MERR. ET PERRY AGAINST *STAPHYLOCOCCUS AUREUS*

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Abstract - The prevalence of Gram-positive *Staphylococcus aureus* in infectious diseases is increasing in Vietnam for recent years. Therefore, novel class of antibiotic or active compounds is indispensable to treat this common worldwide pathogen. In the present study, we investigated the antibacterial activities of *Cleistocalyx operculatus* (Roxb.) Merr. et Perry leaves collected in Quang Nam. The results showed that ethanolic crude extract and three fractions partitioned in different polar solvents suppressed *Staphylococcus aureus* ATCC 6538. The ethyl acetate and n-hexan fractions exhibited a significant inhibitory effect, with minimum inhibitory concentrations (MIC) values of 0.1 mg/ml and 0.2 mg/ml, respectively. And flavonoids was possibly the active substances responsible for the antimicrobial activity of this medical plant. These initial findings are the premise of further studies for the treatment of infection. It also provides the scientific evidences for the traditional utilization of *Cleistocalyx operculatus* (Roxb.) Merr. et Perry leaves.

Key words - *Staphylococcus aureus*; *Cleistocalyx operculatus*; antimicrobial activity; flavonoid

1. Introduction

Staphylococcus aureus (S. aureus) is a major cause of clinical infections in human. These Gram-positive bacteria have been causing a series of diseases such as minor skin infections, cellulitis, folliculitis, scaly skin syndrome, abscesses, pneumonia, meningitis, osteomyelitis, mac endocarditis, sepsis. The mortality rate is quite high in invasive *S. aureus* infection [1]. The application of antibiotics in treatment regimens has effectively eliminated many species of pathogens. However, with the extensive use or even abuse of broad-spectrum antibacterial drugs, resistance to antimicrobial agents has been increasing [2]. A more worrying issue is that *S. aureus* has become resistant to antibiotic therapy. Nam LV *et al* has studied the antibiotics profile of *S. aureus* strains isolated from bloodstream infection patients in Northern Vietnam, in which resistance against penicillin, erythromycin and clindamycin were 100%, 65.1% and 60.5%, respectively [3]. Thus, it is important to discover new substances that could overcome the infectious diseases caused by *S. aureus.*

For centuries, medical plants have been used as traditional treatment of microbial infection. And many bioactive compounds extracted from these plants have been approved to have antibacterial activity against *S. aureus* [4], [5], [6]. *Cleistocalyx operculatus* (Roxb.) Merr. et Perry (*C. operculatus*), a member of Myrtaceae family, is widely distributed in China, North and Central regions of Vietnam, Malaysia, and other tropical countries. Its leaves have been commonly used as herbal tea for gastrointestinal disorders or as folk medicine against skin infection. *In vitro* and *in vivo* various biological activities such as anticancer, antitumor, anti-inflammatory of *C. operculatus* leaves and buds have been examined [7], [8], [9]. Methanolic extract from leaves of *C. operculatus* exhibited inhibitory activity mainly against *Streptococcus mutans* that causes dental decay [10]. The essential oil from *C. operculatus* leaves (CLO) showed the antibacterial effect against *S. aureus* and supportive effect on burn wound healing. Treatment of this CLO fully recovered second-degree burn wound in mice after 20 days, whereas saline treated burn wound formed uneven epidermal layer with necrotic ulcer [11]. In our previous study, both aqueous and ethanolic extracts of *C. operculatus* leaves collected in Quang Nam could inhibit the growth of Gram-positive bacteria *S. aureus* and *Listeria monocytogenes* but not with Gram-negative *E.coli* or *Salmonella* sp [12].

The reach and extraction of the secondary metabolites from medical plants are a major focus of investigation. Solvents used for the extraction process are reported to have an influence on the nature and the amount of these compounds extracted from herbal. For example, polar solvents are used to extract phenolic compounds, their glycosides, saponins, and non-polar solvents are used for the extraction of fatty acids and steroids. Many studies have shown the impact of different solvents on the presence of secondary metabolites and/or their biological activity [13], [14].

In present work, we further investigate the effects of three fractions of crude ethanolic extract of *C. operculatus* leaves against *S. aureus*. All fractions exhibited inhibitory properties against the growth of this pathogen. The ethyl acetate fraction was found to have the highest activity with minimum inhibitory concentration (MIC) 0.1mg/ mL. This fraction and n-hexane fraction contain more flavonoids than n-butanol fraction. These results were the first screening to develop new antimicrobial drugs from *C. operculatus* harvested in Quang Nam.

2. Methods

2.1. Plant material and extraction

C. operculatus fresh leaves were harvested in Dien Ban, Quang Nam, Vietnam, in September 2021 and identified at University of Medicine and Pharmacy, VNU. Crude extraction using ethanol was previously done [12].

Briefly, dried leaves powder (1 kg) was extracted three times with ethanol 70 % (5 L) at 25ºC for 72 h. The liquid extract was then filtered through Whatman No 4 filter paper (110 mm diamter, 20-25 µm pore size) and evaporated under pressure in rotary evaporator (EYELA, Japan) at 50ºC until complete dryness. After that, the ethanol extract was partitioned with decreasing polar solvents - ethyl acetate, n-butanol and n-hexane with the ratio of 1:5 (w/v) for 24 h to yield respective solvent fractions. These separated extracts were kept at 4ºC until required for antimicrobial testing. All fractions were dissolved in Dimethyl sulfoxide (DMSO) 10% to 100 mg/ml and then diluted to the corresponding concentration in Meuller Hinton Broth medium (MHB - Himedia).

2.2. Microorganisms

Staphylococcus aureus ATCC 6538 was kindly provided by Quatest 2, Danang city, Vietnam. The bacteria were maintained on Meuller Hinton agar (MHA – Himedia), at 37 ºC under aerobic condition.

2.3. Agar well diffusion method

This method is widely used to evaluate the antibacterial activity of plants or microbial extract. *S. aureus* were cultured overnight in MHB at 37 ºC, with shaking at 200 rpm, followed by diluting to achieve turbidity equivalent to 0.5 Mcfarland (approximately 10^8 CFU/mL). 100 µL of this bacterial suspension was spread on MHA plate using sterile swab. Then, a hole with a diameter of 9 mm was punched aseptically with a tip, and a volume (100 µL) of extract solution at desired concentration was introduced into the well. Ampicillin (30 µg/mL) and DMSO 10% were used as positive and negative control, respectively. Then, plates were kept at 4ºC for 2 h so that incubated the substances can evenly diffuse in medium agar, followed by overnight incubation for 24 h, at 37ºC. The diameters of inhibition growth zones are measured after the incubation period [15].

2.4. Determination of Minimum Inhibitory Concentrations (MIC)

MIC was determined by resazurin-based turbidometric (TB) assay. Stock solutions of the extracts were prepared in 1.5 mL microcentrifuge tubes (Eppendorff) by dissolving dry plant extract in dimethyl sulphoxide (DMSO) to a final concentration of 100 mg/mL. The two-fold serial dilutions from the stock solution were made ranging from 50 mg/ mL to 0.05mg/mL using MHB in 96-well microtiter microplates. The bacterial suspension containing approximately 10^6 colony-forming units/mL (CFU/mL) was prepared from a 24 h culture with shaking at 37ºC. 50 µL of this suspension and 50 µL of diluted extract solution were inoculated into each well. The negative control wells housed the broth media and the extracts of different concentrations; another well contained only *S.aureus* and DMSO. The positive control wells were a combination of bacteria suspension and ampicillin (30 µg/mL). After overnight incubation at 37°C, 30 µL of a 0.015% of resazurin solution (Santa Cruz, USA) was added to each well as an indicator of microbial growth. The colour change was then assessed visually. The lowest concentration prior to color change was considered as the MIC value [16]. The assay was made in duplicate.

2.5. Determination of Minimum bactericidal Concentrations (MBC)

MBC was recorded as the lowest concentration of tested extracts that can inhibit 99.9% of the bacteria after 24 h incubation at 37 °C. Briefly, 100 µL taken from the well obtained from the above MIC experiment (MIC value) and three or four wells above the MIC value well were subcultured on MHA plates. The number of colonies was counted after overnight incubation at 37°C. The concentration of sample that inhibits more than 99% of the initial inoculum was considered as MBC value [17]. Each experiment was repeated at least three times.

2.6. Total flavonoid content

The total flavonoid content in the fractions was determined using spectrophotometric method, which is based on the formation of a flavonoid-aluminum complex. Briefly, 0.5 mL of sample was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 1M potassium acetate, 0.1 mL of 10% aluminum chloride and 2.8 mL of distilled water, followed by incubating at room temperature for 30 minutes. Then, samples were measured the absorbance at wavelength 415 nm by UV–vis spectrophotometer. Quecertin (QE) was used as a reference standard flavonoid to make calibration curve. The flavonoid content of each fraction was calculated in terms of QE equivalent (mg QE/g of extract) using an equation obtained from the standard quercertin graph [18], [14].

2.7. Statistical analysis

Experiments were carried out in triplicate and results were expressed as mean ± SD (standard deviation). Microsoft Excel 2016 was used to carry out statistical analysis.

3. Results and discussion

The flower buds ("Nu Voi") and leaves of *C. operculatus* ("La Voi") have been commonly used to make beverage for long time. The water extract of *C. operculatus* buds was shown to decrease the frequency of contraction in an isolated rat heart perfusion system. This herbal also has strong protective effects on lipid peroxidation in rat. Previous phytochemical reports have characterized the phytochemical such as oleanane-type triterpenes and flavonoids isolated from *C. operculatus* [19]. However, *C. operculatus* leaves harvested in Quang Nam province have not been well-studied until now.

3.1. Antibacterial activity of C. operculatus crude extract and separated fractions

In this study, the antimicrobial properties of ethanolic extract (EE), and its fractions at different concentrations were assessed by agar diffusion method. The results revealed that all crude extract of *C.operculatus* leaves and ethyl acetate (EA), n-hexane (HE) and n-butanol (BU) fractions are efficiently suppressing the growth of *S.aureus* with variable potency. As stated in Figure 1 and Table 1, EA fraction showed a maximum zone of inhibition against this tested pathogen (17.5 ± 0.71 mm). Both EA and HE fractions exhibited stronger inhibition than crude extract, while BU had less activity (11.0 ± 0.00 mm). NT Dung reported that ethanolic extract of *C. operculatus* buds could inhibit the growth of entire tested Gram-positive bacteria, include *S.aureus*. The diameter of inhibition zone were measured in the range of 9.0 to 15.0 mm [20].

C. operculatus essential oil also exhibited antibacterial activity *against S. aureus* (7.17 ± 0.12 mm) whereas they had no activity against *Pseudomonas aeruginosa* [11]. Thus, our data confirmed the biological effect of ethanolic extract and fractions from three different solvents of *C. operculatus* leaves.

Table 1. *Inhibition zone of crude extract and fractions against S. aureus ATCC 6538*

Extracts/Fractions	Zone of inhibition (mm)	
	100 mg/ml	50 mg/ml
Ethanolic extract (EE)	15.5 ± 0.71	10.5 ± 0.70
Ethyl acetate fraction (EA)	17.5 ± 0.71	14.5 ± 0.71
n-hexane fraction (HE)	16.5 ± 0.70	14.0 ± 0.00
n-butanol fraction (BU)	11.0 ± 0.00	9.00 ± 0.70

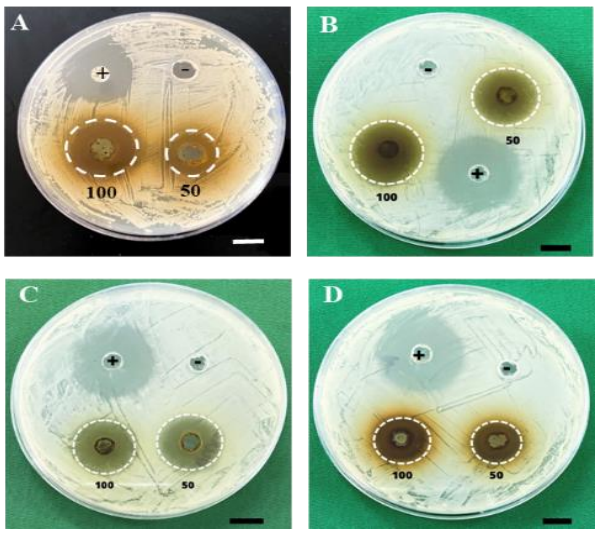

Figure 1. *Antimicrobial activity of ethanolic extract (A), ethyl acetate fraction (B), n-hexane fraction (C), and n-butanol fraction (D) at 100 mg/ml (100) and 50 mg/ml (50) against S. aureus. Ampiciline (30 µg/ml) and DMSO 10% were used as positive (+) and negative (-) control, respectively. Scale bar 1 cm*

3.2. Minimum Inhibitory Concentrations (MIC) value of different fractions from ethanol extraction of C. operculatus

Next, ethanolic extract and its EA, HE and BU separated fractions were examined to identify their minimum inhibitory concentration (MIC) values by using the resazurin assay. In this assay, active bacterial cells reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink), giving a direct quantifiable measure of bacterial metabolic activity [21]. As shown in Figure 2, all ethanolic extract and its fractions were classified as active antimicrobial agents as their MIC values ranging from 0.1 to 0.5 mg/ml [22]. The crude extract and n-butanol fraction revealed weaker activity with MIC of 0.4 mg/ml, compared to the ethyl acetate and n-hexane fractions with MIC of 0.1 mg/ml, 0.2 mg/ml respectively. Among these extracts, the EA separated fraction demonstrated the most effective fraction suppressing tested *S.aureus*.

Solvents commonly used in extraction of medicinal plants are polar solvent (e.g., water, alcohols), intermediate polar (e.g., acetone, dichloromethane), and nonpolar (e.g., n-hexane, ether, chloroform). In our study, we used three solvents – ethyl acetate, n-butanol and n-hexane, with different polarity to separate fraction from crude extract. Ethyl acetate is widely used as a buffer for extraction of bioactive compound because of its chemical and biological functions such as medium polarity (relative polarity = 0.228) and minimum cell toxicity. It can help to extract both polar and non-polar compounds [23]. According to Nguyen et al [24], *C. operculatus* and *Morus alba* ethyl acetate extract (CM) has strong beneficial effect on dextran sulfate sodium (DSS)-induced acute colitis in mice. The CM extract significantly reduced a series of cytokines such as TNF-α, IL-1β, IFN-γ and infiltration of neutrophils that involved in inflammation on colitis [24]. N-hexane (relative polarity = 0.009), nonpolar solvent, is

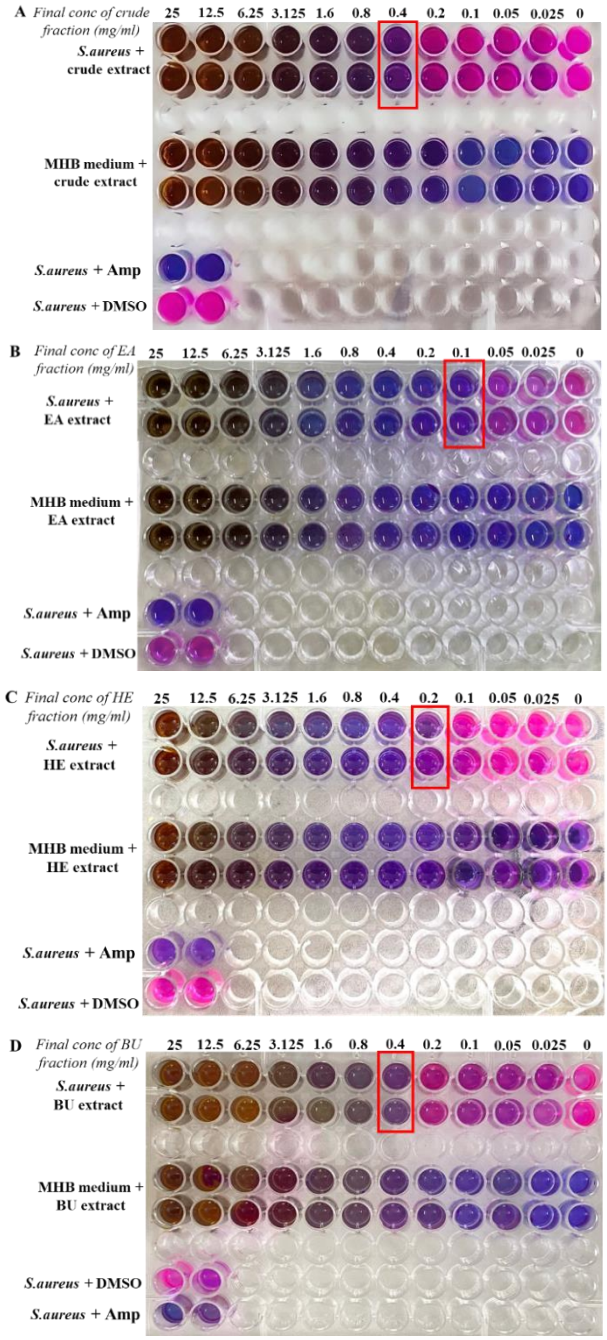

Figure 2. *Resazurin-based 96-well plate microdilution method to determine MIC value of crude extract (A) and three different fraction – etyl acetate (B); n-hexane (C) and n-butanol (D)*

the preferred chemical for oil extraction. The essential oils of *C. operculatus* leaves collected by hydrodistillation were found to possess an antimicrobial effect against *Bacillus cereus* and *S.aureus* [11], [25]. N-butanol has been used as a renewable biogenic extraction solvent for isolating polar products [26]. Rebeca et al showed that n-butanol fraction from *Enantia chlorantha* stem bark has significant activity on all tested bacteria with MIC values ranging from 32 to 256 µg/ml [4]. The n-butanol fraction of *Caesalpinia sappan* L. also exhibited antibacterial effect against S.aureus and MRSA with diameters of 16.44 - 17.77 mm [27]. In our study, we also select n-butanol as solvent to partitioned from ethanolic extract. However, this n-butanol fraction has weaker activity compared to others. These results are considered to be due to different classes of phytochemicals separated by different solvents and herbal material. Compounds extracted from medical plants are mainly dependent on the types of chemical solvents used in the extraction and fractionation processes.

3.3. Minimum Bactericidal Concentrations (MBC) value of the ethyl acetate fractions of C. operculatus

The ethyl acetate fraction which had the highest antibacterial activity was chosen for further examinations. The MBC is complementary to the MIC, which demonstrates the lowest level of tested agent resulting in microbial death. And the respective minimum concentration of this fraction capable of inactivating 99.9% *S. aureus* present was 0.4 mg/ml (Figure 3). If the ratio MBC/MIC ≤4, the effect was considered as bactericidal but if the ratio MBC/MIC > 4, the effect was defined as bacteriostatic. Thus, *C. operculatus* EA fraction in this study was identified as a bactericidal agent as the ratio of the MBC to the MIC value is 4 [17]. This fraction also exhibited the expected inhibition against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) isolated from the clinical samples (unpublished data).

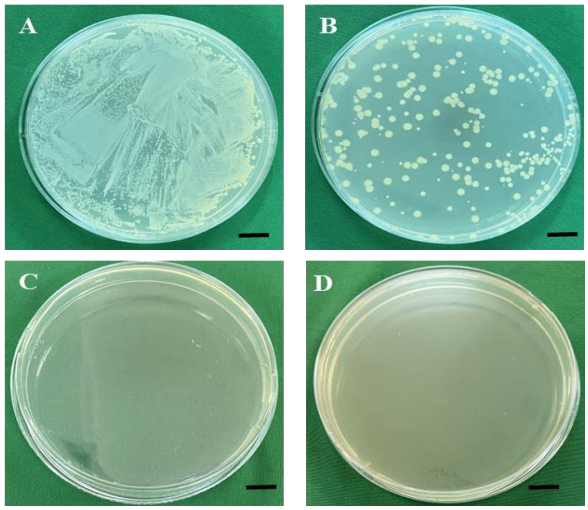

Figure 3. *MBC value determined by plating broth with various ethyl acetate concentration range from 0.1 mg/ml to 0.8 mg/ml. (A)-EA 0.1 mg/ml; (B)-EA 0.2 mg/ml; (C)-EA 0.4 mg/ml; (D)- EA 0.8 mg/ml. Scale bar 1cm*

3.4. Determination of flavonoid content in different separated fractions

Extraction of medicinal plants is a process of separating active components or secondary metabolites such as alkaloids, flavonoids, terpenes, saponins, steroids, and glycosides from inert or inactive material using an appropriate solvent and standard extraction procedure. [28]. We quantified the flavonoid contents of EA, HE and BU fractions by using quercetin (QE) as standard compound (Figure 4A). All three fractions have presence of flavonoid, of which the higher contents were 83.0 ± 0.63 mg QE/g and 86.3 ± 1.03 mg QE/g of samples in EA and HE extracts, respectively (Figure 4B).

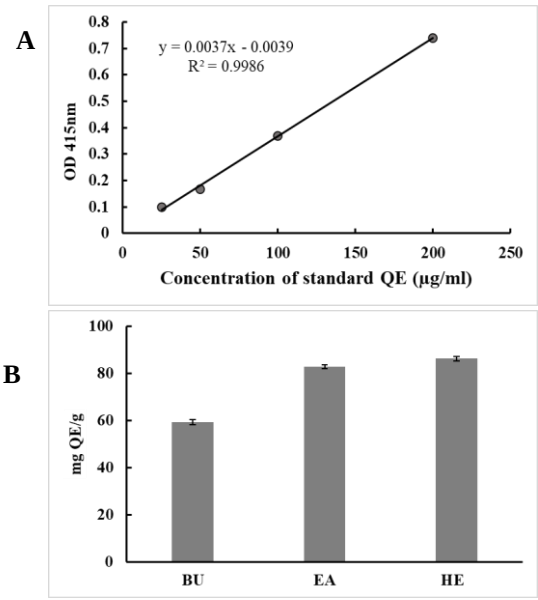

Figure 4. *Total flavonoid contents (TFC) of three fractions from C. operculatus ethanolic extract. (A) – Calibration curve of standard Quercetin for TFC determination. (B) – TFC of n-butanol (BU), ethylacetate (EA) and n-hexan (HE) fractions expressed as mg QE/g*

Flavonoids are the class of natural phenolic compounds synthesized in plants which are responsible for the characteristics of flavor, color, and pharmacological activities. They are potent antioxidants protecting plants from unfavorable environmental conditions. Many previous reports showed that flavonoids provide different medicinal properties such as antiallergic, antioxidant, anti-inflammatory, hepatoprotective, antiviral, antiproliferative, and anticarcinogenic activities. Several thousand flavonoids have been identified such as quercetin, epigallocatechin gallate (EGCG), myricetin, etc. Moreover, the molecular mechanisms of plant-derived flavonoids against bacteria have already clarified. Flavonoids can inhibit the bacterial cell envelop synthesis, efflux pump, biofilm formation or quorum sensing. They also target to the virulent factors, acid nucleic or Adenosine triphosphate (ATP) synthesis [29]. Phuong et al (2016) revealed the presence of flavonoids and terpenes in the methanolic extract of *C. operculatus* leaves by thin-layer chromatography (TLC) [10]. Several flavonoids with chalcone skeleton have been isolated from the buds and the leaves of C. operculatus showed significant inhibitory effects on the viral neuraminidases from two influenza viral strains, H1N1 and H9N2 [30]. Among these compounds, 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) was reported to induce apoptosis on human pancreatic cancer cells PANC-1 and MIA PACA2 [7].

4. Conclusion

In our present study, we demonstrate the strongly suppression *S. aureus* of EA and HE separated fractions. Flavonoids is suggested the main component contributed to the antibacterial potency in these extracts. However, another phytochemical probably cannot be excluded. The ethyl acetate and n-hexane fractions of *C. operculatus* leaves could be an interesting material for the development of drugs or functional foods using in infectious diseases. Isolation of bioactive constituents from these fractions to identify the desired pharmacological agents as well as a mechanism of action of potential compounds should be done in the future.

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