Herbal Extract as an Adjunctive Therapy to Reverse Antibiotic Resistance in *Staphylococcus aureus* through Inhibition of Biofilm and Increased Cell Permeability

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Abstract

Staphylococcus aureus presents a substantial risk to public health since it can acquire antibiotic resistance, facilitated by biofilm formation and altered cell membrane permeability. In our study, we observed substantial biofilm production in S. aureus strains, exhibiting reduced sensitivity to kanamycin (Km), especially in Km-adapted strains. Herbal therapy offers a promising solution to address antibiotic resistance. Extracts from Andrographis paniculata (AP), Eucalyptus globulus (EG) and Zingiber zerumbet (ZZ) effectively inhibited biofilm formation and reduced cell surface hydrophobicity in both wild-type and Km-adapted strains.

Additionally, extracts from Plectranthus amboinicus (PA), Clerodendrum inerme (CI), Combretum quadrangulare (CQ), EG, AP and ZZ increased S. aureus cell permeability, enhancing antibiotic sensitivity. These findings suggest herbal extracts could serve as adjunctive treatments for S. aureus infections by targeting biofilm formation and cell membrane properties, potentially reversing antibiotic resistance and reducing required antibiotic dosages for effective disease management.

Keywords: Antibiotic-resistant, Biofilm formation, Cell permeability, *S. aureus.*

Introduction

Staphylococcus aureus, a ubiquitous bacterium found on the skin and mucosal surfaces of humans and animals, can cause a range of infections from mild to severe, including pneumonia, endocarditis and sepsis[4,8,13,20,24]. One of its notable characteristics is its ability to develop resistance to multiple antibiotics including methicillin and other beta-lactams[2]. Methicillin-resistant *S. aureus* (MRSA) strains pose significant therapeutic challenges and have become a leading cause of mortality globally[2], with infections affecting over 200 nations and territories as of 2019[26].

The sluggish rate of novel antibiotic development contrasted with the rapid emergence of antibiotic-resistant bacterial strains and has raised global apprehension, prompting investigations into herbal medicine as a potential therapeutic solution[33]. Several mechanisms by which herbal remedies interfere with *S. aureus* have been demonstrated, including disrupting the bacterial cell membrane[35], inhibiting biofilm formation and quorum sensing[21], suppressing essential enzymes, inhibiting antioxidant systems[5], reducing the expression of efflux pumps[14], disturbing the cytoplasmic membrane[34], increasing the permeability of the membrane[12] and interfering with the metabolic network[17] and demonstrating synergistic effects when combined with conventional antibiotics[16].

Disrupting bacterial biofilm formation has become a viable strategy for the treatment of *S. aureus* infections [3, 25, 37]. Biofilms consist of bacteria organized in a self-produced extracellular polymeric matrix, serving as a physical barrier that protects them from host immune defenses and antimicrobial agents [32]. In the biofilm matrix, *S. aureus* cells exhibit increased antibiotic resistance up to 1000 times higher than their planktonic counterparts[22]. Various herbal extracts, including carvacrol, limonene and citral, have been found to effectively inhibit biofilm formation in *Listeria monocytogenes*, *Escherichia coli* and *S. aureus*, thereby enhancing their susceptibility to antibiotics[1,9,10].

Our previous study demonstrated the antibacterial activity of certain Vietnamese herbal plant extracts against *S. aureus* and its antibiotic-adapted variants, without inducing further drug resistance [27]. Building upon these promising findings, the present study aimed to investigate the potential of these herbal extracts as supplementary agents to traditional antibiotics. We investigated whether combining the extracts with antibiotics could increase the sensitivity of antibiotic-resistant strains to the antibiotics. Additionally, we endeavored to elucidate the putative mechanisms by which these extracts might sensitize *S. aureus*, including the modulation of biofilm formation and alterations in cell membrane permeability. Our findings could contribute to the advancement of alternative therapeutic strategies to address antibiotic resistance in *S. aureus* by enhancing the efficacy of existing antibiotics through synergistic interactions with herbal adjuvants.

Material and Methods

Preparation of bacterial strains: The bacteria were provided by NTT Hi-tech Institute laboratory (Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam). *S. aureus* ATCC 25923 was referred to as a wild-type (WT) strain while its laboratory-evolved counterpart adapted to

kanamycin, referred to as a kanamycin adapted strain (Km-adapted)[27]. Prior to conducting the antibiotic resistance assays, the strains were spread onto tryptic soy agar (TSA) (Himedia, India) and incubated for 24 h at 37°C to obtain single colonies. Subsequently, a single colony was transferred to 5 mL of TSB culture medium and incubated with shaking at 130 rpm and 37°C for 24 h to obtain exponentially growing cultures.

Preparation of herbal extracts: The herbal extracts were obtained from NTT Hi-tech Institute (Nguyen Tat Thanh University, Vietnam), including *Andrographis paniculata* (AP), *Clerodendrum inerme* (CI), *Combretum quadrangulare* (CQ), *Eucalyptus globulus* (EG), *Plectranthus amboinicus* (PA) and *Zingiber zerumbet* (ZZ). They were extracted from their respective leaves, stems, or rhizomes using diethyl ether and lyophilized following previously study [27]. The extracts were solubilized in DMSO (Sigma-Aldrich, USA) to a concentration of 50 mg/mL and stored at -20°C.

Protocol for the development of *S. aureus* biofilms: *S. aureus* overnight cultures in TSB medium were centrifuged to collect bacterial cells, which were then resuspended in Mueller-Hinton broth (MHB) (Sigma-Aldrich, USA) at concentration of 1×10^7 CFU/mL (OD_{600} = 0.1). Subsequently, 200 µL samples were introduced into 96-well plates and placed in an incubator set at 37°C. The control groups consisted solely of MHB medium without any bacterial inoculation. Planktonic cells were removed from the plates after 8, 16, 24, 32 and 40 h of incubation. Following this, the plates underwent triplicate washes with phosphate-buffered saline (PBS).

Afterwards, the plates were left to dry in the air for 2 h. Following that, they were stained with a crystal violet solution (1 mg/mL) for 30 min. Following the staining process, the plates underwent two rounds of washing with PBS to eliminate any surplus dye. Subsequently, the crystal violet was dissolved using 33% acetic acid. The quantification of biofilm formation was conducted by measuring the absorbance at 595 nm in comparison to the control groups[29].

Examination of the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC), of herbal extracts and Kanamycin on *S. aureus*: The MIC of plant extracts and kanamycin (Km) against *S. aureus* was determined by microdilution with resazurin (Sigma-Aldrich, USA) as a colorimetric indicator[27]. Briefly, 100 µL of 1×10^7 CFU/mL of *S. aureus* (OD_{600} = 0.1) was mixed with 100 µL of serial 2-fold dilutions of Km or extracts in MHB to 200 µL in 96 well plates. The group that contained MHB only was assigned as the control group. After 24 h incubation at 37°C, MICs were detected by identifying the lowest concentration that retained the resazurin blue color. For MBC determination, 50 µL of cultures showing no visual growth were plated on MHA. MBCs were determined by observing the absence of bacterial colonies on MHA after 24 h of cultivation at the lowest concentration.

Examination of the minimum biofilm eradication concentration (MBEC) of Km on biofilm-resident *S. aureus*: Biofilm-forming *S. aureus* was cultured in MHB with or without herbal extracts in 96-well plates at 37°C for 24 h. The planktonic cells were eliminated and washed twice with PBS, then the Km was introduced at varying concentrations for 24 h at 37 °C. Subsequently, biofilms were rinsed and detached by pipetting and the viability of *S. aureus* was assessed by drop-plating on TSA. The MBEC was determined as the minimum concentration of Km that effectively eliminated biofilm colonies[7].

The impact of herbal extracts on the formation of biofilms in *S. aureus*: *S. aureus* overnight cultures in TSB medium were centrifuged to collect bacterial cells, which were then resuspended in MHB to 1×10^7 CFU/mL (OD_{600} = 0.1). Within 96-well plates, 200 µL portions of the bacterial culture were exposed to sub-inhibitory concentrations (1/8 to 1/2 × MIC values) of different herbal extracts for 24 h to facilitate the formation of biofilm. The biomass of biofilm was then quantified employing a crystal violet (CV) assay[29]. The biofilm-resident and planktonic bacteria were collected and their sensitivity to Km was assessed using the microdilution assay. The results were reported using MBEC, MIC, MBC respectively.

Investigation of the hydrophobicity of the *S. aureus* cells surface: The effect of herbal extract at sub-inhibitory concentrations on *S. aureus* surface hydrophobicity was evaluated by a chloroform adhesion assay [6]. After being treated with herbal extract at concentrations below the inhibitory threshold for 24 h, *S. aureus* cells were collected, rinsed twice and resuspended in PBS until their optical density (OD600) reached approximately 0.3 (A1). One ml of *S. aureus* suspension was mixed with an equal volume of chloroform, vortexed for 2 min and allowed to stand at 25 °C for 30 min. The OD_{600} of the aqueous phase (A2) was measured. The formula was used to determine the hydrophobicity index (I) as follows:

$$I = (1 - A2/A1) \times 100\%$$

Measurement of bacterial cell permeability of *S. aureus*: The crystal violet (CV) assay was used to measure the change in *S. aureus* membrane permeability [11]. *S. aureus* overnight cultures in TSB medium were centrifuged to collect bacterial cells, which were then resuspended in MHB to 1×10^7 CFU/mL (OD_{600} = 0.1), supplement with or lacking herbal extracts at a concentration of 1/2 MIC for 24 h.

The bacterial cells were collected by centrifugation at 10000 × g at 4 °C for 2 min, then suspended in 0.1 g/ml CV in PBS. The suspension was subsequently subjected to centrifugation at 1000 × g for 15 min to collect the

supernatant and detected OD_{590}. The OD value of 0.5 mM was regarded as 100%. The quantification of crystal violet uptake was expressed as a percentage as follows:

$$\text{Percentage uptake of crystal violet} = \frac{\text{OD value of CV solution} - \text{OD value of sample}}{\text{OD value of CV solution}} \times 100$$

Statistical Analysis: The experiment was conducted using a completely randomized design (CRD) with 3 replicates for each treatment. The results were calculated by averaging three repeated measurements and presented as the mean ± standard error of the mean. Analyzing the data involved the use of SAS 9.4 software from SAS, Inc., located in Cary, NC, USA. Statistical significance was assessed by comparing groups using both the T-test and Tukey's test. A significance level of less than 0.05 was employed to establish statistical significance.

Results and Discussion

Biofilm formation ability of *S. aureus*: The development of biofilm by both strains of *S. aureus* was observed for 40 h using crystal violet staining. The results, depicted in fig. 1A, revealed strong biofilm forming capacities for both strains, with the wild-type (WT) strain displaying considerably greater biofilm biomass than the Km-adapted strain. In addition, an examination of the growth curves (Figure 1B) indicated that the cell densities of both *S. aureus* strains reached their highest point 24 h of cultivation. Subsequently, they transitioned into the stationary phase and steadily declined after 32 hours of incubation. Therefore, a 24 h of incubation was assigned for future investigation.

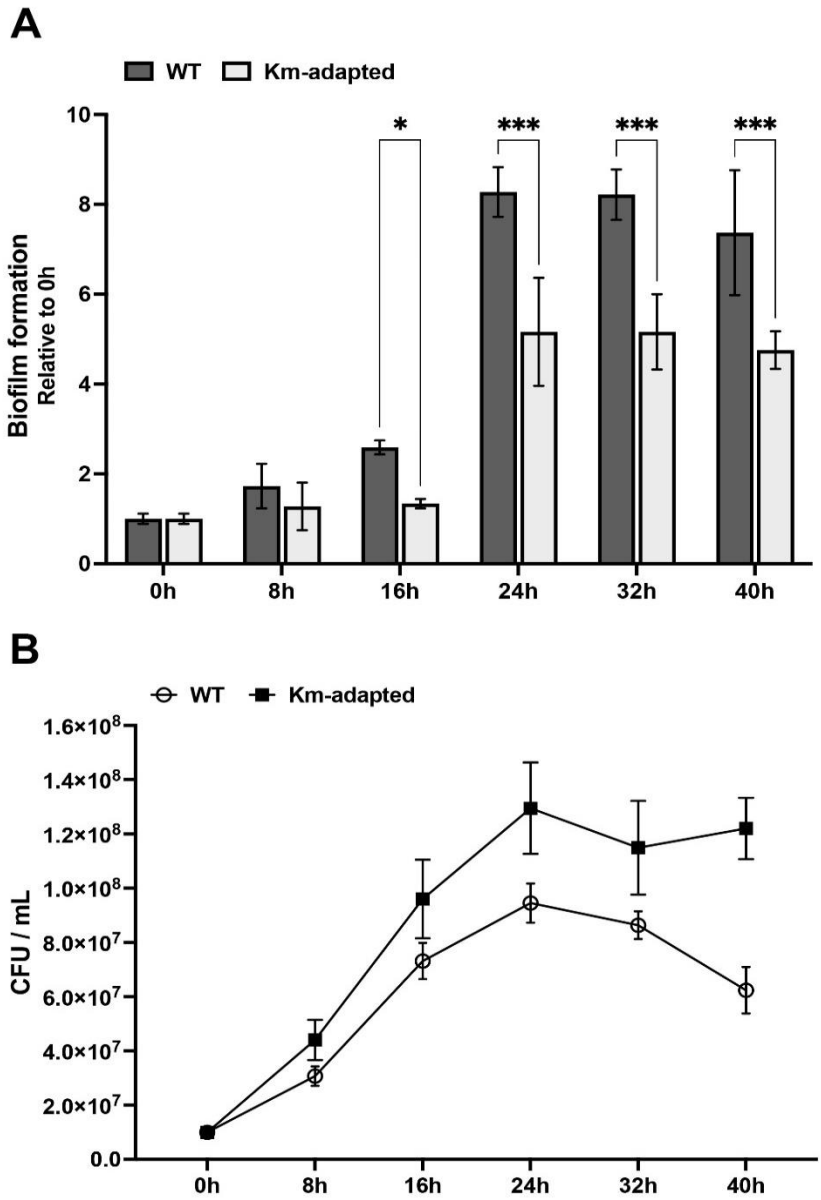

Fig. 1: (A) Biofilm mass production and (B) the growth curve of *S. aureus* at different times interval. WT: wild-type strain; Km-adapted: Kanamycin-adapted strain. * Indicates significant differences compared between Km-adapted and WT groups, p < 0.05

Effect of biofilm on the viability of *S. aureus* under antibiotic exposure: Biofilm formation by *S. aureus* contributes to increased antibiotic resistance and can result in treatment failures and chronic persistent infections [30]. The extracellular polymeric matrix creates a physical barrier, impeding antibiotic penetration and access to bacterial cells within biofilms. Furthermore, biofilm-associated bacteria often display altered metabolic states and physiologies that reduce their susceptibility to antimicrobial agents [32]. Our data revealed that *S. aureus* cells residing within biofilms exhibited remarkable tolerance, requiring a 2-fold higher concentration of Km to achieve elimination compared to their planktonic counterparts (Table 1).

This disparity in susceptibility was further amplified for the Km-adapted strain, where biofilm localization conferred an extraordinary 800-fold increase in resistance, necessitating Km concentrations up to 20,000 µg/mL for complete elimination, in stark contrast to the WT strain (Table 1). These findings corroborate the previous observations by McLaughlin et al[23], thus highlighting the protective nature of biofilms against antibiotic eradication of *S. aureus*.

Effect of herbal extract at subinhibitory concentration on the biofilm formation of *S. aureus*: The effect of herbal extracts to biofilm formation in *S. aureus* was initially investigated by determining their MICs against both strains. Notably, the MIC values of these extracts were comparable for the WT and kanamycin-adapted strains (Table 2). Subsequent investigation utilized sub-inhibitory concentrations of each extract to evaluate their ability to impede the development of biofilm in *S. aureus* over 24 h. Our data demonstrated that the extracts of ZZ, EG and AP resulted in a reduction in biofilm production by both strains of *S. aureus*, dependent on the dosage administered (Fig. 2D, E, F).

On the other hand, the presence of CQ, CI and PA extracts did not have any impact on biofilm formation (Fig. 2A, B, C). Our results agreed with previous studies indicating that the extract derived from *A. paniculata* (Burm f.) and *Z. zerumbet* (L.) might reduce the biofilm formation in *P. aeruginosa*[15,19]. These herbal extracts could potentially serve as natural alternatives to conventional antibiotics for managing biofilm-associated *S. aureus* infections.

Effect of herbal extract at subinhibitory concentrations on the cell surface hydrophobicity of *S. aureus*: The hydrophobic nature of the bacterial cell surface is essential for its capacity to create biofilms. To investigate whether the herbal extracts modulated this property, a chloroform adhesion assay was performed[32]. The findings in fig. 3 demonstrated that the surface hydrophobicity of *S. aureus* cells was considerably reduced when exposed to sub-inhibitory doses of ZZ, EG and AP extracts. This reduction in surface hydrophobicity was found to be directly related to the observed decrease in biofilm formation. This decrease disrupts the connections between bacterial cells and adhesion sites, inhibiting the initial attachment and subsequent formation of biofilms[31].

However, the treatment of CQ, PA, or CI extracts did not cause any changes in the hydrophobicity of the cell surface, align with the non-inhibitory effect on *S. aureus* biofilm formation. Our findings consistent with examination have shown that different plant extracts, such as limonene, citral, carvacrol and extracts might inhibit the formation of *S. aureus* biofilms by decreasing the hydrophobicity of cell surfaces[31,36]. Thus, the herbal extracts from ZZ, EG and AP showed promising potential for the effective treatment of biofilm-associated *S. aureus* infections via interference with cell surface hydrophobicity.

Effect of herbal extract at subinhibitory concentrations on the efficacy of Km to eliminate the biofilm-resident *S. aureus*: Previous studies showed certain plant extracts can inhibit bacterial biofilm formation and can increase antibiotic sensitivity[1,9,10]. Therefore, the hypothesis that herbal extracts could increase the antibiotic sensitivity of *S. aureus*, was also evaluated in this study.

Table 1
MICs, MBCs and MBECs of Km on *S. aureus*

	WT				**Km-adapted**		
	Planktonic		**Biofilm**		**Planktonic**		**Biofilm**
	MIC	MBC	MBEC		MIC	MBC	MBEC
Km (µg/mL)	6.25	12.5	25 #		2500 *	10000 *	20000 *#

WT: wild-type strain; Km-adapted: Kanamycin-adapted strain. * Indicates significant differences compared between the MIC, MBC and MBEC values of kanamycin for Km-adapted and WT strains. # Indicates significant differences compared between the Km concentration required to eradicate biofilm-reside *S. aureus* (MBEC) and planktonic *S. aureus* (MBC).

Table 2
MIC values of herbal extracts on *S. aureus*

	MIC (µg/mL)					
	CQ	**ZZ**	**CI**	**PA**	**EG**	**AP**
WT	1250	250	625	2500	625	625
Km-adapted	1250	250	625	2500	625	625

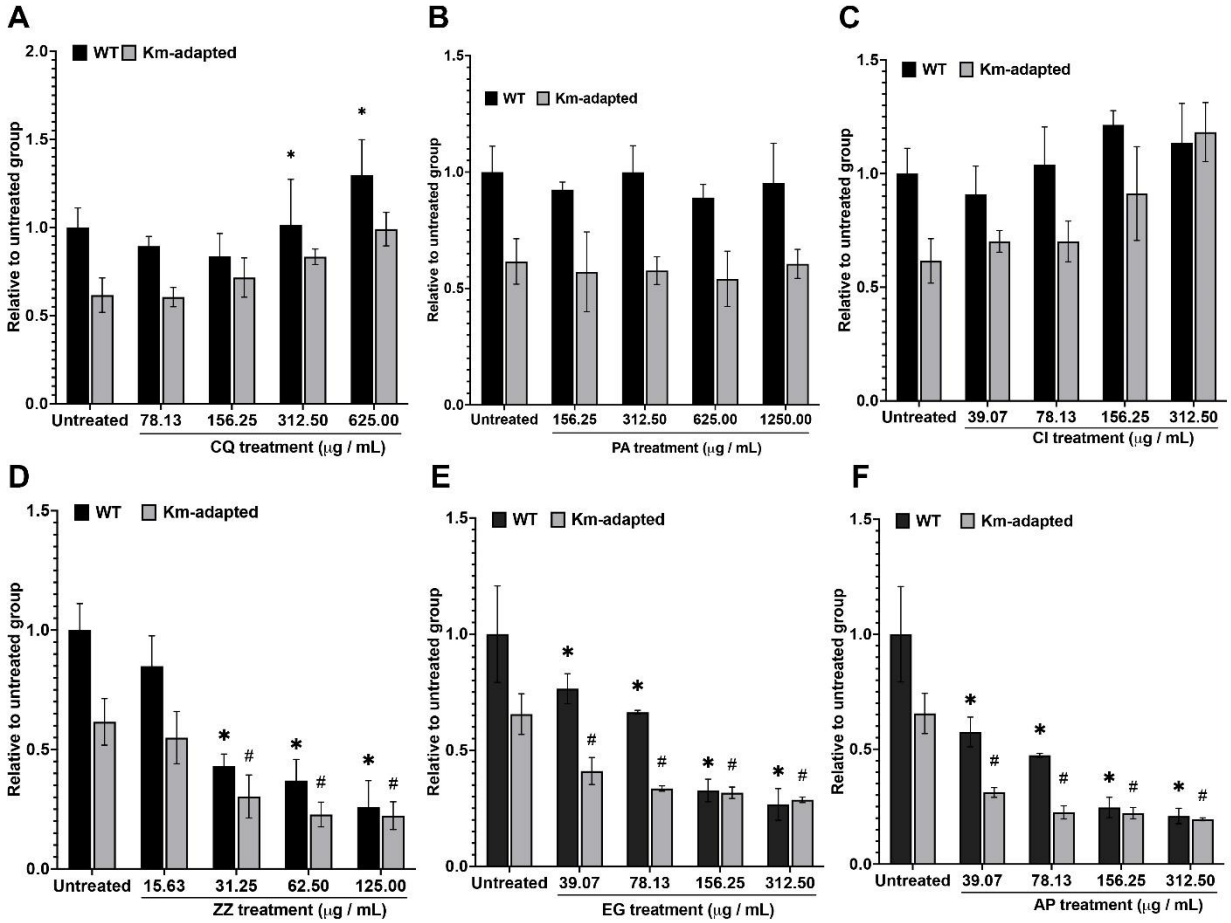

Fig. 2: Effect of herbal extracts at different concentrations on the biofilm formation of S. aureus.
WT: wild-type strain; Km-adapted: Kanamycin-adapted strain. * Indicates significant differences compared with the untreated group, $p < 0.05$

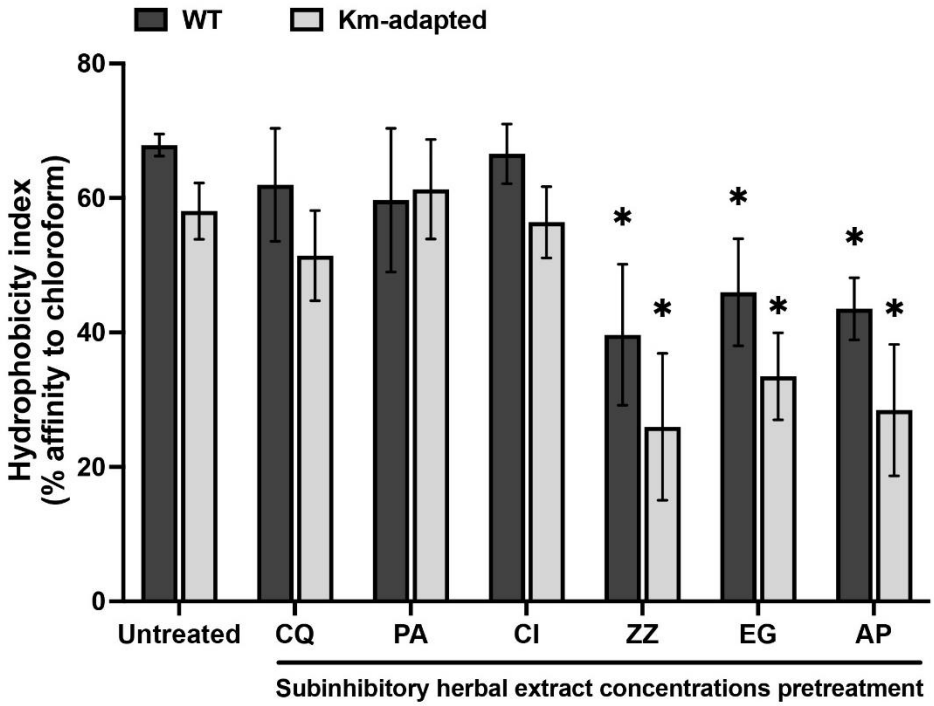

Fig. 3: Effect of sub-inhibitory herbal extract concentrations on the cell surface hydrophobicity of *S. aureus*.
* Indicates significant differences compared with the untreated group, $p < 0.05$

Surprisingly, the sensitivity of both *S. aureus* strain to Km increased by pretreatment of AP (312.5 µg/mL) or ZZ (125 µg/mL) (Table 3). Notably, the MBC of Km to eradication of Km-adapted strain was reduced by 16-fold from 10,000 to 625 µg/mL, following AP pretreatment (Table 3). A significant decrease in antibiotic resistance was observed among the biofilm-residing Km-adapted strains, as indicated by the reduced MBEC of Km required to eliminate *S. aureus* compared to the untreated control group (Table 3). Furthermore, the extracts of CQ, PA and CI did not directly prevent the biofilms development of *S. aureus*. However, they did enhance the susceptibility of both the wild-type and Km-adapted strains to Km, regardless of whether the cells were in a planktonic or biofilm-associated state.

A notable decrease in the MBEC value for the Km-adapted *S. aureus* strain was observed with the AP extract at 312.5 µg/mL, led to a remarkable 16-fold reduction, bringing the value down from 20,000 µg/mL to 1,250 µg/mL, even in the presence of a biofilm. This discovery is especially promising given the sluggish progress in developing new antibiotics. It underscores the potential of using herbal extract pre-treatment as a valuable strategy to tackle the increasing problem of antibiotic resistance.

Effect of herbal extract at subinhibitory concentrations on bacterial cell permeability of *S. aureus*: Bacteria have evolved several strategies to evade the effects of antibiotics such as altering the peptidoglycan layer and membrane permeability, thereby to diminish the antibiotic effectiveness[28,38]. We therefore further investigated whether herbal extract affects cell permeability. The findings presented in fig. 4 indicate that the Km-adapted strain cell permeability was lower compared to the wild-type strain, indicated by the decrease of crystal violet uptake, which is consistent with the adaptive profile of antibiotic-resistant strains[28]. The cell permeability of the Km-adapted strain exhibited an increase following treatment with herbal extracts, in comparison to the untreated group.

Additionally, a corresponding decrease in resistance to Km was observed, as depicted in fig. 4. Previously, several compounds, including chelerythrine and rhodomyrtosone B, have been shown to modify membrane permeability and ion channels, thereby decrease antibiotic resistance in *S. aureus*[12,18]. These findings provide scientific evidence that supports the potential utilization of herbal treatments alongside antibiotics for treating infectious disease caused by *S. aureus*.

Table 3
MICs and MBCs of Km on planktonic *S. aureus* and MBEC of Km on biofilm-resident *S. aureus* after 24 h co-incubation with different sub-inhibitory herbal extract concentrations.

		WT				Km-adapted		
		Planktonic		Biofilm		Planktonic		Biofilm
		MIC	MBC	MBEC		MIC	MBC	MBEC
	Untreated	6.25	12.50	25.00		2500.00	10000.00	20000.00
Subinhibitory herbal extract concentrations pretreatment	CQ (µg/mL)							
	312.50	3.13 *	6.25 *	12.50 *		625.00 *	5000.00 *	5000.00 *
	625.00	3.13 *	6.25 *	12.50 *		625.00 *	2500.00 *	5000.00 *
	ZZ (µg/mL)							
	62.50	1.56 *	6.25 *	12.50 *		625.00 *	2500.00 *	5000.00 *
	125.00	1.56 *	6.25 *	12.50 *		312.50 *	1250.00 *	2500.00 *
	CI (µg/mL)							
	156.25	6.25 ns	6.25 *	12.50 *		2500.00 *	2500.00 *	5000.00 *
	312.50	6.25 ns	6.25 *	12.50 *		2500.00 *	2500.00 *	5000.00 *
	PA (µg/mL)							
	625.00	6.25 ns	6.25 *	12.50 *		1250.00 *	2500.00 *	5000.00 *
	1250.00	6.25 ns	6.25 *	12.50 *		1250.00 *	1250.00 *	2500.00 *
	EG (µg/mL)							
	156.25	3.13 *	6.25 *	12.50 *		1250.00 *	5000.00 *	10000.00 *
	312.50	3.13 *	3.13 *	6.25 *		625.00 *	5000.00 *	10000.00 *
	AP (µg/mL)							
	156.25	1.56 *	6.25 *	12.50 *		625.00 *	1250.00 *	2500.00 *
	312.50	1.56 *	3.13 *	12.50 *		312.50 *	625.00 *	1250.00 *

WT: wild-type strain; Km-adapted: Kanamycin-adapted strain. * Indicates significant differences compared between the MICs, MBCs and MBECs of Km on *S. aureus* in herbal extracts pretreatment groups and the untreated group, $p < 0.05$

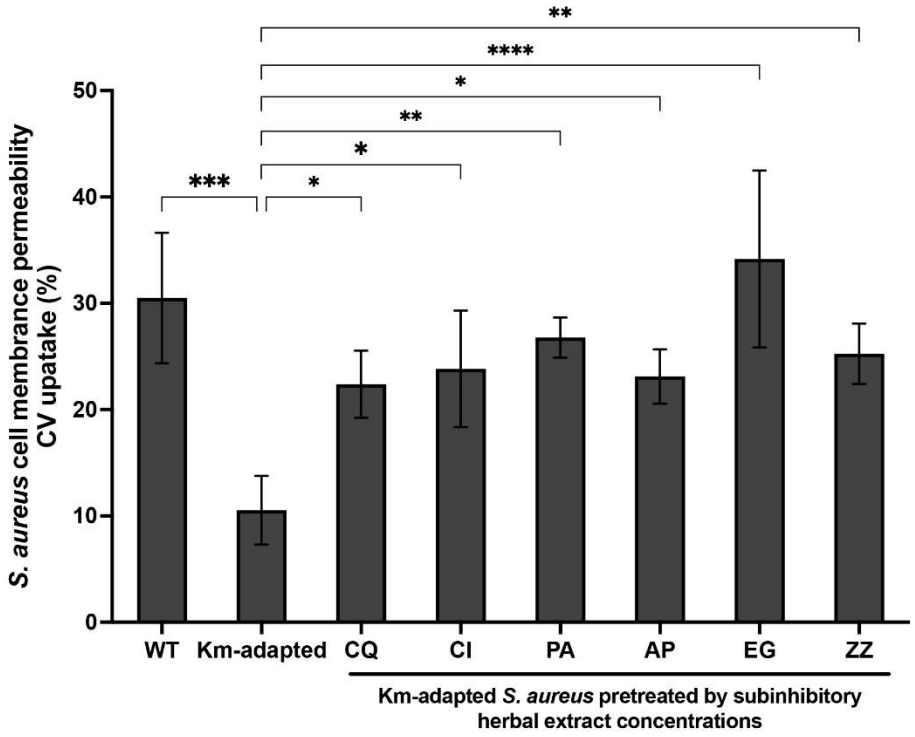

Fig. 4: Effect of subinhibitory herbal extract concentrations change in S. aureus membrane permeability (assayed by crystal violet uptake) in the presence of different herbal extracts.
WT: wild-type strain; Km-adapted: Kanamycin-adapted strain. * Indicates significant differences compared with the Km-adapted group without pretreated with herbal extracts, $p < 0.05$

Conclusion

To summarize, the issue of antibiotic resistance in *S. aureus* presents a substantial obstacle, exacerbated by the development of biofilms and alterations in cell membrane permeability. The hydrophobic interactions between bacterial cells and biotic or abiotic surfaces are essential in promoting the formation of biofilms. This study showed that extracts derived from *Plectranthus amboinicus*, *Eucalyptus globulus* and *Andrographis paniculata* can reduce hydrophobicity, which prevents the formation of biofilms and restores the susceptibility of *S. aureus* antibiotic-resistant strains.

The results of our study indicate that these extracts have the ability to partially reverse kanamycin resistance in both planktonic and biofilm-associated *S. aureus*. This is likely achieved by enhancing the permeability of the bacterial membrane and optimizing the antibiotic's accessibility. Integrating these herbal extracts shows potential for effectively treating *S. aureus* infections. However, additional research is necessary to assess their safety, effectiveness and the processes by which they work before they can be used in clinical applications.

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