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Combating multidrug-resistant *Staphylococcus aureus* isolated from camel milk with extracts of *Eucalyptus globulus* and *Calotropis procera*, and their potential role in modulation of resistance to beta-lactam drugs

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Summary

This study aimed to evaluate the efficacy of *Eucalyptus globulus* and *Calotropis procera* against MDRSA and their potential role in modulating resistance to beta-lactam drugs. The study used a total of n = 263 camel milk samples as well as aqueous and ethanolic extracts of both plants from the Cholistan Desert and the Suleman Mountain Range in Pakistan. Different concentrations of the two plants (25, 12.6, 6.25, and 3.125 mg/mL), either alone or in combination with each other, were tested against MDRSA by well diffusion and broth dilution methods. Further, the efficacy of both plant extracts in combination with cefotaxime (30 µg) and ampicillin (1 µg) was also tested. The results indicate that the zone of inhibition (ZOI) of *E. globulus* alone and in combination with *C. procera* was nearly 3 times as large as that of *C. procera*. Ethanolic extracts of the two plants plants, alone or in combination with either of the two antibiotics, had a significantly ($P < 0.05$) larger ZOI than their aqueous extracts did. Further, the modulation factor for the drugs in combination with ethanolic extracts of both plants exhibited strong synergy ($P < 0.05$), and the minimum inhibitory concentration (MIC) of ethanolic extracts was also significantly lower than that of the aqueous extract of either plant ($P < 0.05$). In conclusion, the ethanolic extracts of *E. globulus* and *C. procera* alone and in combination are effective against MDRSA and can play a role in modulating resistance to beta-lactam antibiotics.

Keywords: drug resistance, plant extracts, *Staphylococcus aureus,* beta-lactams

Antibiotic resistance has become a serious global issue that affects even treatable strains of bacteria (6). An international multicenter study of antimicrobial resistance of *Staphylococcus aureus* (*S. aureus*) reported the occurrence of methicillin-resistant *S. aureus* (MRSA) is low (< 1%) in hospitals of Northern Europe, moderate (6~22%) in middle European countries, the United States, New Zealand, and Australia, and very high (28~63%) in Southern European countries, as well as in some parts of the United States, Asia, and South Africa (8, 26). This worldwide escalation of bacterial resistance to conventional medical antibiotics is a serious health concern for modern medicine and causes thousands of deaths. Hence, there is an immediate need to develop new approaches for handling this life threatening problem (5).

To overcome the resistance to antibiotics, it is first necessary to understand its physiological pathways

and underlying mechanisms. Major mechanisms of antibiotic resistance are the efflux of the antibiotic from the bacterial cell through efflux pumps, enzymatic modification or degradation of the antibiotic molecule, and alteration of the antibiotic target, which prevents the antibiotic from binding and, thus makes it lose its activity (13). These mechanisms suggest the use of different approaches, such as taking advantage of synergistic activity between antibiotics and non-antibiotics, inhibition of resistance enzymes that degrade or modify the antibiotic to a non-active form, blocking antibiotic efflux, or facilitating antibiotic entry into the cell and altering the physiology of insensitivity to the antibiotic in cells (25). However, the high prevalence of resistant bacteria hinders the application of these strategies and thus necessitates the development of alternate approaches that should be safe, effective, and economical.

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. In rural areas of the developing countries, they continue to be used as the primary source of medicine (9). About 80% of people in developing countries use traditional medicines for their health care (15). The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals. Approximately 60% of pharmaceuticals used against infectious diseases and tumors are believed to have been derived from natural sources (21). The increasing resistance of many common pathogens to currently used therapeutic agents has led to renewed interest in the discovery of novel anti-infective compounds. Promising results obtained by using plant extracts as antibiotics justify their increased utilization as a source of pharmaceutical products. As there are approximately 500,000 plant species occurring worldwide, of which only 1% have been phytochemically investigated, there is a great potential for discovering novel bioactive compounds (18). Plant extracts should be investigated for their potential impact on bacterial infection alone and in combination with antibiotics facing resistance.

Eucalyptus globulus (*E. globulus*) consists of citronellol, p-cymene, 1, 8-cineole, citronellyl acetate, limonene, aromadendrene, alloocimene, linalool, beta pinene, gamma terpinene, and alpha terpinol. 1, 8-cineol (eucalyptal) has been reported to have various pharmaceutical activities, including a strong antibacterial activity (19). The antibacterial activity of *Calotropis procera* (*C. procera*) extracts is due to its active ingredients that create pores in cell walls with resultant leakage of cytoplasmic contents, inhibit the electron transport chain, and interfere with the biosynthesis of sphingolipids. These extracts may be used wherever the pathogens are causative agents. Moreover, these plant extracts are used to combat inflammation. *C. procera* has long been used against gastrointestinal tract infections (GIT) in animals and humans as well. The synergistic interaction of plant extracts with antimicrobials has been found to modify the phenotype of resistant bacterial strains to the point that they become susceptible to drugs to which they were formerly resistant (23). However, the synergistic effect of combinations of *E. globulus* and *C. procera* with antibiotics against multidrug-resistant *Staphylococcus aureus* (MDRSA) is still unexplored.

In this study, we investigated the efficacy of aqueous and ethanolic extracts of *E. globulus* and *C. procera* alone and in combination with antibiotics against MDRSA and assessed their effectiveness in the modulation of resistance to beta-lactam drugs. Our study paves the way to the utilization of these plant extracts in combination with antibiotics against those pathogens that can infect animals as well as humans. Hence the findings could be useful in treating humans as well as animals for MDRSA. However, there is still a need to explore the molecular basis and detailed mechanisms of the activity of these extracts against MDRSA.

Material and methods

Milk sample collection and isolation of multiple drug-resistant *S. aureus*. A total of n = 362 camel milk samples were collected from two camel-populated areas: the Cholistan Desert (n = 185) and the Suleiman Mountain range (n = 177) in Pakistan. The samples were screened for subclinical mastitis with the California mastitis test (CMT), and all CMT-positive samples (n = 362) were subjected to isolation and identification of *S. aureus* according to guidelines from Bergey's Manual of Determinative Bacteriology (12). Briefly, milk samples were centrifuged for 10 minutes, and sediments obtained were swabbed on blood agar and incubated at 37°C for 24 h. After incubation, the colonies were sub-cultured on mannitol salt agar for 24 h at 37°C. Round and pinpoint colonies on fermented media were subjected to biochemical characterization by Gram staining, catalase test, coagulase test, and other methods. Mannitol fermentation positive, Gram positive, coagulase positive, and catalase positive colonies were identified as *S. aureus*. These *S. aureus* colonies were further validated by the reference strain "*Staphylococcus aureus* subsp. *aureus*" (ATCC® 25923™). The activated growth (fresh growth during 24 hours of incubation) of *S. aureus* isolates adjusted at 1~1.5 × 10^8 CFU/mL (0.5 McFarland) was examined for susceptibility against a wide range of antibiotics according to (4). Antibiotics, including oxacillin (1 µg), ampicillin (10 µg), cefoxitin (30 µg), vancomycin (30 µg), trimethoprim (25 µg), amikacin (30 µg), oxytetracycline (30 g), gentamicin (10 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), cefixime (5 µg), chloramphenicol (30 µg), enoxacin (10 µg), and streptomycin (10 µg), were aseptically applied on Mueller-Hinton (MH) agar swabbed with *S. aureus*. Antibiotic discs and antibiotic powder used in this study were purchased from Oxoid™ of Thermo Scientific™ through a vendor. Zones of inhibition (ZOI) of the bacteria were measured with Vernier calipers after incubation at 37°C for

24 h and compared with standards provided by the Clinical Laboratory Standards Institute (24). Isolates resistant to more than two classes of antibiotics were considered as multidrug resistant *S. aureus*.

Collection of plants and extraction of active ingredients. Leaves of *C. procera* and *E. globulus* were collected from the Cholistan Desert and the Suleman Mountain Range, respectively. Plants were washed three times with distilled water to remove debris and dried at room temperature without exposure to sunlight. Dried leaves were cut into small pieces and ground to powder. Aqueous and ethanolic extracts were prepared using the Soxhlet apparatus as reported previously (1). Briefly, 100 g of powdered leaves was placed in the thimble of the apparatus at the top of a conical flask containing 500 mL of solvent. The solvent was heated for 12 h (4). The extracts were dried in a rotary evaporator to a semi-solid state and stored at 4°C in dark-colored glass bottles sealed with a cork and aluminum foil until use.

Selection of isolates. Ten MDRSA were selected for assessing their susceptibility to the plant extracts alone and in combination, and for use in drug resistance modulation trials. The selected isolates were resistant to more than two classes of antibiotics, in addition to penicillin and cephalosporin groups.

Determination of zones of inhibition by well-diffusion assay

Plant extracts alone and in combination. The selected MDRSA were adjusted to 1×10^8 CFU/mL by spectrophotometry. MH agar was prepared according to recommendations, seeded with test organisms, and left to solidify. A 4 mm sterile steel borer was used to create an evenly spaced pattern of wells in the media and sealed with MH agar. Stock solution was prepared from dried stocks of plant extracts. Extract (0.1 gram) was dissolved in 1 mL of dimethylsulphoxide (DMSO). Sterilized Eppendrof tubes (1.5 mL) were first pre-weighed on a weighing balance. The extracts were vortexed vigorously until complete dissolution. In a pilot study, the lowest concentration showing a zone of inhibition was 3.125 mg/mL. Using this concentration as a baseline, concentrations twice as high were calculated in order to finally use 25 mg/mL, 12.6 mg/mL, 6.25 mg/mL, and 3.125 mg/mL of both of aqueous and ethanolic extracts of *E. globulus* and *C. procera*. The combination of plant extracts comprised 50% concentration from each plant. The bacteria were incubated with plant extracts at 37°C for 18-22 h, and ZOI was measured with Vernier calipers.

Plant extract in combination with antibiotics. The ethanolic extracts of both plants at 25 mg/mL, 12.6 mg/mL, 6.25 mg/mL, and 3.125 mg/mL were combined with 1 µg of ampicillin or 30 µg of cefotaxime, while the dose of the antibiotic was kept constant in each combination. The plant extract/antibiotic combinations were poured into the wells of MH agar seeded with test organisms, incubated at 37°C for 25 h, and ZOI was measured. Drug resistance modulation was recognized when the ZOI of a drug and plant combination was larger than that of the drug alone.

Determination of minimum inhibitory concentration

Plant extract alone. The minimum inhibitory concentration (MIC) of plant extracts at 25 mg/mL, 12.6 mg/mL, 6.25 mg/mL, and 3.125 mg/mL was estimated by the broth dilution method (24). The optical density (OD) value was measured at zero time of incubation and after 24 hours of incubation at 37°C. The inhibition of growth was measured as the net OD value of the test well (with bacteria and antibiotic/plant extract) less than or equal to the cut-off value.

In combination with β-lactam. In this study we further evaluated the antibacterial activity of the beta-lactam group (ampicillin and cefotaxime) on the basis of a well-diffusion assay. The aqueous and ethanolic extracts were compared by ZOI, and the extract with the higher ZOI was used in the further experiment. The checkerboard protocol was applied by the broth micro-dilution method in a 96-well titration plate to find the range of ampicillin concentrations (0.488--1000 µg/mL) with serial dilutions of plant extracts (10, 5, 2.5, 0.125, 0.625, 0.3125, 0.15625, and 0.078125 mg/mL). OD measured by an ELISA reader at 690 nm wavelength was used to determine growth inhibition.

Statistical analysis. The activity of plant extracts and the modulation of antibiotic resistance were analyzed by one-way analysis of variance (ANOVA), along with Tukey's test as a post-hoc test, using SPSS v. 21 (IBM NY, USA). Statistical significance was set at $P < 0.05$. The modulation factor of drug resistance was calculated as the effect of the drug alone/the effect of the drug in combination with a plant extract.

Results and discussion

***Zone of inhibition of multi-drug resistant S. aureus* with plant extracts alone and combined.** The study found a direct relationship between ZOI and the increasing concentration of aqueous and ethanolic extracts of *C. procera* and *E. globulus* used either alone or in combination (Tab. 1). The ZOI against MDRSA was significantly larger ($P < 0.05$) for *E. globulus* than

Tab. 1. Zone of inhibition (mm) of MDR *S. aureus* for single and combined plant extracts

Concentration (mg/mL)	Extract type	*C. procera*	*E. globulus*	Both plants*
25	Aqueous	$6.76^a \pm 0.39$*	$18.1^a \pm 0.88$**	$16.4^a \pm 1.26$***
	Ethanolic	$8.92^b \pm 0.77$*	$24.6^b \pm 0.97$**	$22.4^b \pm 0.70$***
12.5	Aqueous	$5.37^a \pm 0.26$*	$16.6^a \pm 1.08$**	$15.1^a \pm 1.20$**
	Ethanolic	$7.10^b \pm 0.57$*	$21.4^b \pm 1.35$**	$18.6^b \pm 0.126$***
6.25	Aqueous	$4.80^a \pm 0.14$*	$12.3^a \pm 0.48$**	$9.21^a \pm 1.66$***
	Ethanolic	$6.63^b \pm 0.36$*	$18.9^b \pm 0.32$**	$12.25^b \pm 1.98$***
3.125	Aqueous	$4.32^a \pm 0.10$*	$9.3^a \pm 0.73$**	$8.08^a \pm 0.36$***
	Ethanolic	$5.29^a \pm 0.03$*	$15.15^b \pm 0.6$**	$10.16^b \pm 0.58$***

Explanations: * *E. globulus* and *C. procera* were used in combination. Different numbers of asterisks within rows for each concentration and each extract indicate significant difference at $P < 0.05$. Different alphabetical superscripts within columns show significant difference at $P < 0.05$ between aqueous and ethanolic extracts of *C. procera*, *E. globulus,* and *E. globulus* + *C. procera*

Tab. 2. Zone of inhibition of antibiotics in combination with ethanolic extracts of plants against *S. aureus*

Concentration of plant extracts* (mg/mL)	Plants	Ampicillin			Cefotaxime		
		Combination (ZOI, mm)	Modulation factor	Modulated susceptibility	Combination (ZOI, mm)	Modulation factor	Modulated susceptibility
3.125	*C. procera*	16.32 ± 0.39[a]	> 0.5 (0.65)	Resistant	15.83 ± 0.38[a]	> 0.5 (0.70)	Resistant
	E. globulus	29.02 ± 0.41[b]	< 0.5 (0.36)	Sensitive	26.35 ± 0.47[b]	< 0.5 (0.42)	Sensitive
6.25	*C. procera*	19.05 ± 0.64[a]	> 0.5 (0.56)	Sensitive	16.67 ± 0.58[a]	> 0.5 (0.65)	Resistant
	E. globulus	33.05 ± 0.40[b]	< 0.5 (0.32)	Sensitive	30.63 ± 0.61[b]	< 0.5 (0.36)	Sensitive
12.5	*C. procera*	22.50 ± 0.58[a]	< 0.5 (0.47)	Sensitive	18.24 ± 0.69[a]	> 0.5 (0.61)	Sensitive
	E. globulus	36.11 ± 0.36[b]	< 0.5 (0.29)	Sensitive	33.81 ± 0.48[b]	< 0.5 (0.33)	Sensitive
25	*C. procera*	26.03 ± 0.45[a]	< 0.5 (0.41)	Sensitive	20.15 ± 0.78[a]	> 0.5 (0.55)	Sensitive
	E. globulus	39.03 ± 0.43[b]	< 0.5 (0.27)	Sensitive	36.68 ± 0.50[b]	< 0.5 (0.30)	Sensitive

Explanations: * Effective dose of ampicillin (1 µg) and cefotaxime (30 µg) was constant with all concentrations of plant extracts. Zone of inhibition (mm) in combination with plants, ampicillin standard < 17 mm = resistant, > 17 mm = sensitive; cefotaxime standard < 17 mm = resistant, > 17 mm = sensitive; values in parenthesis are actual modulation factors; modulation factor < 0.5 indicates synergy. For each concentration (mg/mL) of extract, different alphabetical superscripts within column between *C. procera* in combination with ampicillin and *E. globulus* in combination with cefotaxime show significant difference at $P < 0.05$. For each concentration (mg/mL) of extract, different alphabetical superscripts within column between *C. procera* in combination with cefotaxime and *E. globulus* in combination with cefotaxime show significant difference at $P < 0.05$; Modulation factor = $ZOI_{drug\ alone}/ZOI_{drug\ with\ plant\ extract}$

it was for *C. procera.* The ZOI for *E. globulus* alone and in combination with *C. procera* were nearly 3 times those of aqueous and ethanolic extracts of *C. procera* alone. Ethanolic extracts of both plants, alone and in combination showed significantly larger ($P < 0.05$) ZOI compared to aqueous extracts at all concentrations, except *C. procera* at 3.125 mg/mL (Tab. 1).

Modulation of the resistance of *S. aureus* to ampicillin and cefotaxime. Ethanolic plant extracts were used in combination with ampicillin and cefotaxime, and we found that ZOI for drugs combined with extracts of both plants at all concentrations were significantly larger ($P < 0.05$) than those for the antibiotics alone (Tab. 2). The ZOI of ampicillin and cefatoxime combined with *E. globulus* (at all concentrations) were nearly 1.8 times as large as those for both antibiotics combined with *C. procera*. Although the combination of *C. procera* at 3.125 mg/mL with ampicillin showed modulation, ZOI remained in the resistant range, that is < 17 mm, whereas for all other combinations, ZOI was > 17 mm (Tab. 2).

All concentrations of *C. procera* and *E. globulus* in combination with ampicillin exhibited a modulation factor < 0.5, except for *C. procera* at 3.125 and 6.25 mg/mL, which had modulation factors > 0.5. Further, except for 3.125 mg/mL, all concentrations of *E. globulus* in combination with cefotaxime showed modulation factors < 0.5. The susceptibility of cefotaxime-resistant *S. aureus* to *E. globulus* at all concentrations was in the sensitive range (> 17 mm). The susceptibility of cefotaxime-resistant *S. aureus* to cefotaxime combined with *C. procera* remained in the resistant range (< 17 mm) for *C. procera* at 3.125 and 6.25 mg/mL, but was in the sensitive range (> 17 mm) for *C. procera* at 12.5 and 25 mg/mL.

MIC of plant extracts against MDRSA. The MIC of the aqueous extract of *C. procera* was significantly

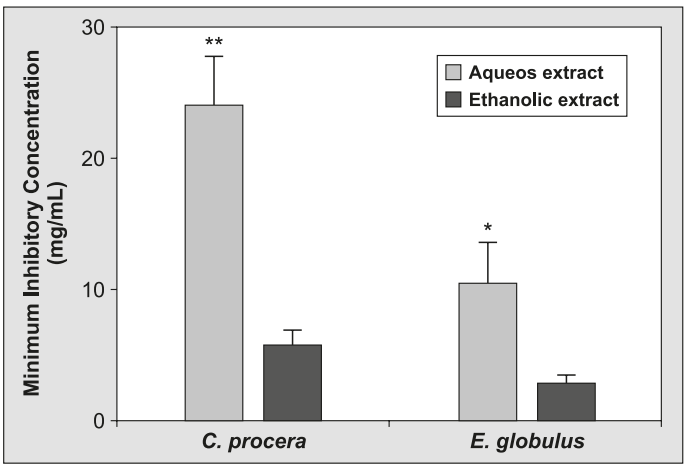

Fig. 1. Comparison of antimicrobial activity of *C. procera* and *E. globulus* in terms of minimum inhibitory concentration (mg/mL) against multi-drug resistant *S. aureus*

higher than that of the ethanolic extract (24.43 ± 3.87 vs 5.94 ± 0.99 mg/mL; $P < 0.05$), and the aqueous extract of *E. globulus* showed a significantly higher MIC than did the ethanolic extract (10.62 ± 3.02 vs 2.97 ± 0.49 mg/mL; $P < 0.05$; Fig. 1).

Modulation of the resistance of *S. aureus* to ampicillin. The MICs of ethanolic extracts (10, 5, 2.5, 0.125, 0.625, 0.315, 0.15625 and 0.078125 mg/mL) in combination with each dilution of ampicillin (1000~0.488 µg/mL) modulated ampicillin resistance (Tab. 3). The combination of ampicillin with *E. globulus* had a significantly lower ($P < 0.05$) MIC against penicillin-resistant *S. aureus* than did ampicillin with *C. procera* at all concentrations except 10 mg/mL. Further, the MIC of ampicillin in combination with the ethanolic extract of *E. globulus* at 0.078125, 0.15625, 0.315, 0.625, 0.125, 2.5, 5 and 10 mg/mL decreased by 55.27%, 75.01%, 88.14%, 92.12%, 92.75%, 97.04%, 97.84%, 98.12%, respectively. The MIC of ampicillin

Tab. 3. Modulation of ampicillin resistant MDR *S. aureus* by ethanolic plant extracts. Minimum inhibitory concentration (µg/mL)

Ampicillin concentration (µg/mL)	Concentration of plant extracts (mg/mL)	Minimum inhibitory concentration of plants (mg/mL)		Modulation factor, % decrease in MIC and susceptibility of *S. aureus*					
				E. globulus			*C. procera*		
		E. globulus	*C. procera*	Modulation factor	*Reduction in MIC of Ampicillin in combination with *E. globulus* (%)	Susceptibility	Modulation factor	*Reduction in MIC of Ampicillin in combination with *C. procera* (%)	Susceptibility
0.488-1000	0.078125	13.28 ± 3.77[a]	29.69 ± 4.94[b]	> 2 (2.24)	55.27	Resistant	< 2 (1)	0	Resistant
0.488-1000	0.15625	7.42 ± 1.23[a]	17.19 ± 4.94[b]	> 2 (4)	75.01	Sensitive	< 2 (1.73)	42.10	Resistant
0.488-1000	0.3125	3.52 ± 0.83[a]	14.84 ± 2.47[b]	> 2 (8.43)	88.14	Sensitive	> 2 (2.001)	50.02	Resistant
0.488-1000	0.625	2.34 ± 0.83[a]	14.06 ± 3.29[b]	> 2 (12.69)	92.12	Sensitive	> 2 (2.11)	52.64	Resistant
0.488-1000	0.125	2.15 ± 0.62[a]	9.38 ± 3.29[b]	> 2 (13.81)	92.75	Sensitive	> 2 (3.16)	68.41	Resistant
0.488-1000	2.5	0.88 ± 0.21[a]	2.34 ± 0.82[b]	> 2 (33.74)	97.04	Sensitive	> 2 (12.69)	92.12	Sensitive
0.488-1000	5	0.64 ± 0.24[a]	1.81 ± 0.46[b]	> 2 (46.39)	97.84	Sensitive	> 2 (16.40)	93.90	Sensitive
0.488-1000	10	0.59 ± 0.21[a]	0.93 ± 0.43[a]	> 2 (50.32)	98.1	Sensitive	> 2 (31.92)	96.87	Sensitive

Explanations: EMIC (µg/mL) for ampicillin alone is 29.69 ± 4.94. Different superscripts within rows indicate significant difference. Values in parenthesis are actual modulation factors. Modulation factor ≥ 2 indicates synergy. Modulation factor = MIC drug/MIC combination drug and plant extract. % Decrease in MIC of drug = (MIC of drug alone – MIC of drug in combination/MIC of drug alone)100

in combination with *C. procera* at 0.078125, 0.15625, 0.315, 0.625, 0.125, 2.5, 5 and 10 mg/mL was reduced by 0, 42.10%, 50.02%, 52.64%, 68.41%, 92.12%, 93.90%, 96.87%, respectively. While modulation factors at concentrations of 0.078125 and 0.15625 mg/mL were < 2, *C. procera* at concentrations of 0.2125, 0.625, 2.5, and 10 mg/mL showed synergistic effects (modulation factors > 2). The MIC of *C. procera* at > 0.125 mg/mL against ampicillin-resistant *S. aureus* was < 8 µg/mL. We found that *E. globulus* had a significantly lower MIC against ampicillin-resistant *S. aureus* than *C. procera* had.

Synergistic effects resulting from the combination of antibiotics with various plant extracts have been studied extensively (25), and it was reported that ethanolic extracts of *E. globulus* were more effective against drug-resistant *S. aureus* than they were against other drug-resistant bacteria (3). Further, fruit of *E. globulus* showed a MIC of 0.12~1 mg/mL against MRSA isolates from clinical infections in humans (17). The lower MIC in our study might be due to differences in the strain of MRSA and the usage of leaf extracts rather than fruit. It is also well-documented that the stage of growth of the plant at collection as well as the extraction solvent and the mode of action of plant extracts against MDRSA also affect the efficacy of plant extracts (19). One mechanism of drug resistance is antibiotic efflux without drug alteration or degradation, which results in reduced intracellular antimicrobial concentration (14). The higher MICs against these strains might be due to the limited efficacy of some extracts and the high resistance of certain strains. However, the findings of the present study are not in agreement with (16), who used aqueous extracts of the latex of *Calotropis gigantea*, which showed a ZOI of 30 mm and a MIC of 62.5 µg/ml against *S. aureus*. This could be explained by the variation in the *Calotropis* species and the use of latex rather than leaves, since it was observed that latex extracts have a higher level of active ingredients than leaf extracts. Another source reports a ZOI of 12.5 mm with an aqueous extract and 11.1 mm with an ethanolic extract of *C. gigantea* against MDRSA (22). The efficacy of ethanolic extracts of *C. procera* against coagulase-negative staphylococci was higher than that of aqueous extracts (20). Moreover, it was also observed that ethanolic extracts showed a higher MIC or no effect against some staphylococcal and enterococcal species (22). However, the weak activity of ethanolic extracts of *C. procera* against *S. aureus* demonstrated by both paper diffusion and broth dilution methods is also well documented (14). The active ingredient of *C. procera* extracts perforates the bacterial cell walls which results in the leakage of cytoplasmic contents, inhibits electron transport, and interferes with sphingolipid biosynthesis (11).

Our results are in agreement with (7), who found, by the agar disk diffusion method, that leaf extracts of *E. globulus* showed a ZOI > 20 mm against *S. aureus*. The antibacterial activity of this plant is due to its components: citronellol, p-cymene, 1,8-cineole, citronellyl acetate, limonene, aromadendrene, alloocimene, linalool, beta pinene, gamma terpinene, and alpha terpinol (19). It is increasingly acknowledged that 1,8-cineole (eucalyptal) also exhibits pharmaceutical activity, as methanol leaf extracts of *E. globulus* were shown to have MIC_{50} and MIC_{90} of 64 and 128 mg/mL, respectively, against *S. aureus* isolates (2). Ethanol is regarded as the optimal solvent for a maximum

extraction of active plant ingredients, as ethanolic extracts were more effective than water and hexane extracts (2). The effectiveness of *C. procera* extracts against bacteria has been found to vary depending on the solvent. However, in a few studies, aqueous extracts showed a larger ZOI than did ethanol and methanol extracts (2). The larger ZOI was attributed to the fact that aqueous solvents are more effective in phytochemical extraction, specifically of tannins, which show bactericidal activity. Tannins in extracts of *C. procera* have been reported to bind with proteins and produce water-soluble contents that damage the bacteria cell membrane (1). The activity of plant extracts is attributed to the presence of active ingredients in extracts, whose functionality depends on the age of the plant, the solvent, the time and geographic area of collection, and extraction methodology (10).

The combined effect of plant extracts and drugs against *S. aureus* is greater than the sum of their individual effects. This could be due to the formation of complexes that effectively protect the bacterial cell wall against lysis (1, 2). Beta-lactam antibiotics act by binding to the cell wall and blocking bacterial penicillin-binding proteins that inhibit cell wall synthesis. Resistant strains impair this binding by genetic mutations (20) or by producing hydrolyzing enzymes (e.g. lactamases) that break the amide bond (5), resulting in the extrusion of cell contents, which reduces the concentration of the antimicrobial substance inside the cell (13). Plant extracts are capable of modifying these mechanisms (22). In summary, the ethanolic extracts of *E. globulus* and *C. procera* alone and in combination are effective against MDRSA and can play a role in modulating resistance to beta-lactam antibiotics. Further, plant extracts in combination of with antibiotics are regarded as effective candidates against MDRSA. However, there is still a need for molecular, *in vivo*, and *in vitro* studies to evaluate their effects on other pathogens.

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