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Disinfectant Activity of *Eucalyptus globulus* Leaves Extracts Against MRSA and ESBL Producing *Pseudomonas aeruginosa* Isolated From Hospital Environment

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ABSTRACT

Increasing resistance of bacterial isolates from hospital surfaces to antimicrobials is worrisome and require urgent intervention. This study aimed to assess the disinfectant potential of *Eucalyptus globulus* leaf extract against Methicillin resistance *Staphylococcus aureus* (MRSA) and extended spectrum beta lactamase (ESBL)L-producing *Pseudomonas aeruginosa* isolated from hospital environments. The plant leaf was extracted using methanol, ethanol, water, ethyl acetate, and hexane solvents using maceration method. The extracts were then screened for alkaloids, flavonoids, saponins, anthraquinones, tannins, glycosides, and steroids using standard methods. Isolates of *S. aureus* and *P. aeruginosa* from various hospital surfaces were subjected to Gram staining and biochemical tests, with MRSA confirmed via cefoxitin susceptibility and ESBL production confirmed via a combined disc test. Confirmed strains were tested against the plant extracts using agar well diffusion. Additionally, the disinfectant potency was compared to phenol, establishing the phenol coefficient (Pc). Results indicated extraction yields of 20.09%, 11.76%, 11.53%, 2.72%, and 0.26% for water, methanol, ethanol, ethyl acetate, and hexane, respectively. The aqueous extract exhibited the highest inhibitory effect against MRSA (20mm-27mm), while the ethyl acetate extract showed moderate inhibition against ESBL-producing *P. aeruginosa* (9mm-12mm). The phenol coefficient for the aqueous extract against MRSA was >1, suggesting greater efficacy compared to phenol, whereas against ESBL-producing *P. aeruginosa*, it was <1, indicating lower efficacy compared to phenol. Overall, the presence of phytochemicals in *E. globulus* leaf extracts contributed to their inhibitory activity against MRSA but showed less effectiveness against ESBL-producing P. aeruginosa.

Keywords: *Eucalyptus globulus*; Disinfectant; MRSA; ESBL-producing *Pseudomonas aeruginosa;* Phenol coefficient

INTRODUCTION

Hospital-acquired infections present a worrisome challenge to healthcare systems worldwide, contributing significantly to the burden of morbidity and mortality, especially in low resource settings. Among the diverse array of pathogens responsible for these infections, multidrug-resistant pathogens such as *Pseudomonas aeruginosa*, Vancomycin-resistant *Enterococcus faecalis*, Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium difficile* are increasingly resistant to conventional antimicrobial treatments, complicating the management of infections and raising concerns about the efficacy of existing therapeutic strategies (de Oliveira *et al.,* 2017). The rise in antimicrobial resistance is multifactorial, with factors such as the indiscriminate use of antimicrobials and disinfectants playing significant roles (Rozman *et al*.,20 21).

Recent studies have highlighted the persistent presence of multidrug-resistant pathogens on hospital surfaces, even following routine

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disinfection procedures (Yusuf *et al*.,2020). This persistence necessitate the urgent need for alternative to conventional disinfectants that are capable of combating resistant surface pathogens (Egbule, 2016; Yusuf *et al*., 2020). In response to this pressing need, researchers have increasingly turned their attention to natural products with antimicrobial and disinfectant properties, exploring their potential as alternatives to traditional chemical disinfectants.

In many cultures, including Nigeria, indigenous plants have long been utilized for their therapeutic properties in the treatment of various ailments, including infectious diseases. *E. globulus*, commonly known as Gum tree or Blue gum, is one such plant that has received interest for its broad-spectrum antimicrobial activity. Studies have documented its effectiveness against both Gram-positive and Gram-negative bacteria, as well as its antifungal, anti-inflammatory, and insecticidal properties (Shala *et al*., 2021). Notably, *E. globulus* contains bioactive compounds such as 8-cineole (Eucalyptol) and α-phellandrene, which have been shown to exhibit potent cytotoxic effects, making them promising candidates for use in antimicrobial formulations (Hayat *et al*.,2015).

It is based on this background the present study seeks to investigate the disinfectant activity of *E. globulus* leaf extract against MRSA and ESBL-producing *P. aeruginosa* strains isolated from hospital environments.

MATERIALS AND METHODS

Collection of Plant Materials

Fresh mature leaves of *E. globulus* were collected from Shango Housing Estate and identified at the herbarium with voucher number GSUH 398 at the Department of Botany, Gombe State University. The leaves were washed, dried under shade for a week, and then crushed into a fine powder using a mortar and pestle.

Extraction and Phytochemical Screening

Extraction of the leaves was performed using maceration method, following the method described by Ravi *et al*., (2019). Briefly, 100g of powdered *E. globulus* leaves were soaked in 1000ml of water, ethanol, methanol, ethyl acetate, and hexane, respectively, and left for 72 hours. The extracts were filtered using Whatman No. 1 filter paper and evaporated to dryness at 40°C. Phytochemical screening of the extracts was conducted to detect alkaloids (Meyer's test), saponins (Ezeonu *et al*., 2016), tannins (Mir *et al.,*2012),steroids (Ezeonu *et al*., 2016), flavonoids (Shayoub *et al.,*2015), anthraquinones (Ramya, 2020), and cardiac glycosides.

Bioassay Studies

Test Isolates

Test organisms used in this study were isolated from various hospital surfaces, which include floor, bed linen, bed rail, door handle, and bedside table in Murtala Muhammad Specialist Hospital. The isolated bacteria are identified using combination of Gram staining, and biochemical tests. MRSA confirmation was checked phenotypically using cefoxitin susceptibility test while ESBL production was confirmed using the combined disc test (CLSI, 2022).

Preparation of Stock Solution of Extract

The stock solution of each extract was prepared according to Habibu *et al*.,(2021)'s method, with modifications. Briefly, 0.4g of each extract was dissolved in 2ml of 20% DMSO to yield a concentration of 200 mg/ml, which was further diluted to obtain concentrations of 100mg/ml, 50mg/ml, and 25mg/ml.

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Inoculum Standardization

A direct colony suspension method was employed, wherein 24-hour-old colonies of each test isolate was dissolved in 2ml of sterile normal saline, and the turbidity was adjusted to match that of a 0.5 McFarland Standard.

Sensitivity Testing

The antibacterial effects of the leaf extracts were determined using the agar well diffusion method as described by Tawfiq et al., (2017), Mueller-Hinton Agar plates were inoculated with the standardized inoculum, and wells were bored using a sterile cork borer. Test extracts at various concentrations already prepared were delivered into the wells using a micropipette, and the inoculated plates were incubated at 37°C for 24 hours. Gentamicin 10µg disc was used as the positive control. The diameter of inhibition zones was measured, and each test was performed in duplicate.

Minimum Inhibitory Concentration (MIC) Determination

MIC was determined using tube dilution method, and Minimum Bactericidal Concentration (MBC) was determined according to Sales *et al.,* (2020).

Minimum Inhibitory Concentration was determined through dilution of the plant extracts with Nutrient broth in 1: 1 ratio. Exactly 1ml of Nutrient broth was added to 1ml of 100mg/ml dilution of the extracts which was used as standard for all the extracts to obtain the dilutions of 50mg/ml and was further diluted to 25mglml, 12.5mglml,6.25mg/ml, 3.125mg/ml. Standard suspension of the test organisms (0.1ml) was added to each tube. The tubes were incubated at 37^0c for 24 hours .The lowest concentration of the extract showing no growth was regarded as the minimal inhibitory concentration (MIC).

MBC was determined by sub culturing tubes from MIC that show no growth on a fresh Nutrient agar, incubated for further 24 hours. Dilution that showed no single bacterial colony was taken as the Minimum Bactericidal Concentration.

Phenol Coefficient Test

The disinfectant effect of *E. globulus* extract was evaluated in comparison with phenol following the method described by Aminu *et al*., 2021), with modifications. Dilutions of phenol (1:95, 1:100, 1:105, 1:110 and 1:115) and of the test disinfectants (Aqueous and Ethyl acetate extract of *Eucalyptus globulus* at 1:100, 1:200,1:300, 1:400 and 1:500)were prepared, and 24-hour broth cultures of ESBL-producing *P. aeruginosa* and MRSA were added. Sub culturing was performed at intervals of 2.5, 5, 7.5, and 10 minutes, and the presence or absence of growth was recorded. The phenol coefficient (Pc) was calculated by dividing the highest dilution of the disinfectant that kills the organisms at 7.5 minute but not at 5 minutes by the highest dilution of phenol that kills the same organism at 7.5minute but not at 5 minutes.

If Pc=1, it indicates that test disinfectant have the same efficiency as phenol. If Pc>1, it indicates that test disinfectant is more potent than phenol and if Pc<1, it shows that the test disinfectant is less potent than phenol (Aminu *et al.*,2021).

RESULTS

The percentage yields of Eucalyptus globulus leaf extracts are presented in Table 1. The aqueous extract exhibited the highest yield at 20.09%, followed by methanol at 11.76%, ethanol at 11.53%, ethyl acetate at 2.72%, and hexane at 0.26%, showing the lowest yield.

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Table 1: Percentage yield of the leaves extract of *E. globulus*

Solvents used for extraction	Percentage yield of extract
Water	20.09
Methanol	11.76
Ethanol	11.53
Hexane	0.26
Ethyl acetate	2.72

In the phytochemical screening of *E. globulus* leaf extracts (Table 2), ethanol extract exhibited a moderate presence of alkaloids with traces of other phytochemicals. Hexane extracts showed a high presence of steroids, whereas saponins, tannins, and glycosides were abundant in the aqueous extract.

Table 2: Phytochemical analysis of *Eucalyptus globulus*

Extracts	Alkaloids	Saponins	Flavanoids	Tannins	Steroids	Glycosides	Anthraquinones
EU E	++	+	+	+	+	+	+
EU M	+	+	+	++	+	++	-ve
EU EA	++	-ve	++	+	++	+	++
EU NH	-ve	-ve	+	+	+++	-ve	-ve
EU AQ	+	+++	-ve	+++	-ve	+++	-ve

Key: + = Low, ++ = Moderate, +++ = High, -ve = Absent, EU = *E. globulus*, E=Ethanol, M =Methanol, E.A= Ethyl acetate, N.H= Hexane, AQ= Aqueous

The concentrations of aqueous extract of *E. globulus*, as depicted in Table 3, demonstrated varying inhibitory effects against MRSA, with 27mm, 25mm, 22mm, and 20mm observed at concentrations of 200, 100, 50, and 25mg/ml respectively. Similarly, ethyl acetate extract of *E. globulus* exhibited inhibition against *P. aeruginosa*, with zones of inhibition measuring 12mm, 10mm, 9mm, and 9mm at concentrations of 200, 100, 50, and 25mg/ml respectively.

Table 3: Antibacterial activity of *Eucalyptus globulus* against MRSA and ESBL producing *Pseudomonas aeruginosa*

Extracts	Zones of Inhibitions (mm)					Positive control (GN)
Ex	Isolates	200 (mg/l)	100 (mg/l)	50 (mg/l)	25 (mg/l)	10µg)
E E	MRSA	25±0.00	23±0.30	20±0.00	18±0.00	32
E M	MRSA	20±0.60	17±0.50	15±0.00	13±0.70	30
E E.A	MRSA	24±0.50	22±0.00	20±0.00	18±0.40	30
E N.H	MRSA	15±0.40	13±0.30	10±0.10	6±0.00	30
E AQ	MRSA	27±0.50	25±0.20	22±0.50	20±0.00	32
E E	ESBL	9±0.00	8 ±0.50	7±0.30	6±0.00	20
E M	ESBL	9±0.50	6±0.00	6±0.00	6±0.00	25
E E.A	ESBL	12±0.50	10±0.00	9±0.20	9±0.10	22
E AQ	ESBL	10±0.50	7±0.00	6±0.00	6±0.00	25
E NH	ESBL	6±0.00	6±0.00	6±0.00	6±0.00	25

E=Ethanol, M= Methanol, E.A=Ethyl acetate, NH= N-Hexane, AQ=Aqueous, ,GN=Gentamicin

The antimicrobial activity of *E. globulus* leaf extracts against MRSA and ESBL-producing *P. aeruginosa* is summarized in Table 4. For MRSA, the MIC ranged from 25-50mg/ml across all extracts, with the lowest MIC value observed for methanol, ethyl acetate, aqueous,

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and n-hexane extracts (25mg/ml). Against *P. aeruginosa*, the MIC value for *E. globulus* extracts ranged from 12.5-25mg/ml. Similarly, the MBC value of *E. globulus* against MRSA ranged from 25-50mg/ml, while against ESBL-producing *P. aeruginosa*, it also ranged from 25-50mg/ml.

Table 4: MIC and MBC of the *Eucalyptus globulus* extracts against MRSA and ESBL producing *P. aeruginosa*

Isolate	E E	E M	Extracts E EA	E NH	E AQ	
S. aureus	50	25	25	25	25	MIC/mg/ml
P. aeruginosa	25	-	25	-	12.5	
S. aureus	50	-	25	-	50	
P. aeruginosa	25	-	50	-	25	MBC/mg/ml

Key: E= Eucalyptus, Ethanol M=Methanol, EA= Ethyl acetate, NH= N. hexane, AQ= aqueous, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration

The Phenol coefficient for the aqueous extract of *E. globulus* against MRSA is illustrated in Table 5. It was determined by comparing the concentration of the extract that killed the organism at 7.5 minutes but not at 5 minutes to the concentration of phenol that achieved the same effect. The coefficient was calculated as 2.

Table 5: Rideal – walker Phenol co-efficient of Aqueous extract of *E. globulus* and phenol against MRSA

Disinfectant	Dilution of disinfectant	Contact time against MRSA(mins)			
Extract		2.5	5	7.5	10
	1/100	-	-	-	-
	1/200	+	+	-	-
	1/300	+	+	+	-
	1/400	+	+	+	-
	1/500	+	+	+	-
Phenol	1/95	-	-	-	-
	1/100	+	+	-	-
	1/105	+	+	+	-
	1/110	+	+	+	-
	1/115	+	+	+	+

Note: + means growth and – means no growth

Rideal walker co-efficient = Dilution of disinfectant that kills in 7.5min but not in 5min

Dilutions of phenol which kills at 7.5min but in 5mins

200\100= 2

Table 6 shows Phenol co-efficient of Ethyl acetate of *E. globulus* against ESBL producing Pseudomonas aeruginosa was calculated as 0.

Table 6: Rideal – walker Phenol co-efficient of Ethyl acetate of *E. globulus* and phenol against ESBL producing *Pseudomonas aeruginosa*

Disinfectant	Dilution of disinfectant	Contact time against ESBL producing *P. aeruginosa*(mins)			
Extract		2.5	5	7.5	10
	1/100	+	+	+	-
	1/200	+	+	+	-
	1/300	+	+	+	-
	1/400	+	+	+	-

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	1/500	+	+	+	-
Phenol	1/95	-	-	-	-
	1/100	+	-	-	-
	1/105	+	+	-	-
	1/110	+	+	+	-
	1/115	+	+	+	-

Note: + means growth and – means no growth

Rideal walker co-efficient = Dilution of disinfectant that kills in 7.5min but not in 5min

Dilutions of phenol which kills at 7.5min but not in 5min

$$\frac{0}{105} = 0$$

DISCUSSION

The result of extraction showed percentage extraction yield of 20.09% , 11.76% for Aqueous and methanolic extract. This result is contrary to the findings of Khare *et al.*(2021) where methanolic extract has the higher yield than aqueous extracts with 17.17% and 13.07% respectively. This may be because polarity of solvents affects the efficiency of extraction where extraction with higher polarity solvents results in higher percentage yield (Terengganu,2023). Ethanol, Ethyl acetate and Hexane extracts had a yield of (11.56%), (2.72%) and (0.26%) respectively.

Qualitative phytochemical screening of Aqueous and Methanol extract of *Eucalyptus globulus*leaves showed the presence of Alkaloids, Glycoside, Tannin and saponin with absence of Anthraquionone which is contrary to the finding of Khare *et al*.(2021) that reported the presence of anthraquionones in methanolic extract. The difference can be attributed to the difference in extraction time (Sadia *et al.,* 2023) and solubility of the phytochemical in the solvent used.thanolic extract showed the presence of all the phytochemicals, as shown in table 2.

The presume MRSA on nutrient agar, mannitol salt agar produce cream, yellow coloured colonies on the media which was positive for catalase test, coagulase, urease, citrate, and negative for oxidase test. ESBL producing *P. aeruginosa* on cetrimide agar produced green colony with flourescence and was positive for catalase, citrate, oxidase while negative for coagulase, urease. Cefoxitin and combined disc test confirmed the presumed isolates to be MRSA and ESBL producing *P. aeruginosa* (CLSI , 2022).

The sensitivity of MRSA and ESBL producing *P. aeruginosa* to *E. globulus* leave extracts using agar well diffusion was determined measuring zones of inhibition formed around the wells containing various concentrations of extracts (Table 3). Aqueous extract of the plant showed best inhibitory activity with zones of 27mm, 25mm, 22mm,20mm.), This result is contrary with that of Maya *et al*., (2019) in which methanolic extract had higher activity than the aqueous extract.

The best antibacterial activity was shown by Ethyl acetate of *E. globulus* against ESBL producing *P. aeruginosa* with 12mm,10mm,9mm,9mm zones of inhibition, this result is contrary to the findings of (Maya *et al*.,2019) where *P. aeruginosa* was the most resistant bacterium to the Eucalyptus leaves extract because it has an outer membrane as Gram negative as part of their cell wall , which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Maya *et al*., 2019) and lower polar solvent have low antimicrobial activity Zeroual *et al*., (2022).

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Absence of turbidity in the dilution tube containing nutrient broth, organism and extract was taken as the lowest concentration of the extract that resulted in this activity (MIC) which ranged from 25-50mg/ml for Eucalyptus extract against MRSA and 12.5-25mg/ml against ESBL producing *P. aeruginosa*. Absence of visible growth on recovery medium (Agar plate) was considered as MBC showing the bactericidal activity of the extracts ranging from (25-50) for both the organisms .

The high activity of the extract against the test isolates in the study was further supported by comparing it with phenol which shows that Aqueous extract of *E. globulus* has a higher phenol coefficient of 2 against MRSA than Pc of Ethyl acetate extract of *E. globulus* against ESBL producing *P. aeruginosa* of 0 as shown in table 5 and 6 . Phenol coefficent of 2 against MRSA indicate that the extract is more effective than phenol because when phenol coefficient > 1,the test disinfectant is more effective than phenol (Aminu *et al*., 2021).

CONCLUSION

The leaves extracts of *E. globulus* showed broad inhibitory activity against both Gram positive (MRSA) and Gram negative (ESBL producing *P. aeruginosa*). The inhibitory activity is attributed to the presence of phytochemical compounds which include alkaloids, tannin, saponins, phenols, flavoinoid, and steriods. The extracts of the plant showed more effectiveness in comparism to phenol against MRSA , hence can be used as an alternative in production of natural based disinfectants against microorganism that thrive on hospital surfaces and thus reducing antimicrobials resistance.

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