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ANTIBACTERIAL ACTIVITY OF *Anonna muricata* (SOURSOP) LEAVES EXTRACTS ON UROGENITAL ISOLATES OF *Enterococcus faecalis* FROM PATIENTS ATTENDING A TERTIARY HOSPITAL IN CALABAR, NIGERIA

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ABSTRACT

Annona (A.) muricata leaves extracts were evaluated for antibacterial activity against urogenital isolates of *Enterococcus (E.) faecalis* obtained from University of Calabar Teaching Hospital, Calabar, Nigeria. Ethanol and aqueous extracts of *A. muricata* leaves were prepared by Soxhlet extraction method, and tested for antibacterial activity against 20 pre-identified isolates of *E. faecalis* using agar-well diffusion method. Minimum Inhibitory Concentration (MIC) of the extracts was determined using broth dilution method. Antibiotic susceptibility of the isolates was determined using Kirby-Bauer disc diffusion method. Both extracts of *A. muricata* leaves produced significant, concentration-dependent, antibacterial activity against *E. faecalis* isolates with MIC of 50 mg/ml (p= 0.0032). The ethanol extract showed a higher efficacy and inhibited more isolates [12(60%)] than the aqueous extract [10(50%)], with a mean inhibition-zone diameter of 16.5 ± 2.3 mm and 15.0 ± 1.0 mm, respectively. The size of the inhibition-zones correlated significantly, in direct proportion, with concentrations of the extracts (p= 0.012). The efficacy of the ethanol and aqueous extracts was lower than that of Ciprofloxacin (90%), Levofloxacin (80%), and Meropenem (65%), but corresponded with that of Cefoxitin (60%) and Gentamicin (55%), respectively. Identification and isolation of specific antimicrobial compounds present in *A. muricata* leaves crude extracts would likely optimize its potency against bacterial pathogens, such as *E. faecalis,* particularly multidrug resistant strains.

KEYWORDS: *Annona muricata* leaf, crude extract, antimicrobial activity, multidrug resistant pathogens, *Enterococcus faecalis*

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most pressing global health challenges of the 21st century that complicates effective management of a wide range of microbial infections, particularly those caused by bacteria. The World Health Organization (WHO) has declared AMR as one of the top ten global public health threats facing humanity (WHO, 2023). This growing concern has prompted intense search for new antimicrobial agents, particularly those derived from natural sources, which may offer novel mechanisms of action with potentials for surmounting the current therapeutic challenges associated with multidrug resistant pathogens.

In the quest for developing novel antimicrobial agents, several plants have emerged as promising sources of bioactive compounds for potential therapeutic applications. One of such plants is *Annona (A.) muricata*, a fruit-bearing tropical tree belonging to the Annonaceae family, commonly known as soursop or graviola. Although soursop is commonly known and valued for its edible fruits globally, other parts of the plant including leaves, bark and seed, have been found useful in traditional medicine for treatment of various human diseases (Coria-Téllez *et al*., 2022). According to Indian lore, the leaves of *A. muricata* have been used successfully for treatment and prevention of arthritis, asthma, bronchitis, biliary disorder, diabetes, heart

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diseases, hypertension, worm disease, liver disorder, malaria, rheumatism, cancer, and many other diseases caused by bacteria, such as pneumonia, diarrhea, skin and urinary tract infections (Kamath, *et al*., 2017).

Phytochemical analysis of *A. muricata* has revealed a rich and complex array of bioactive compounds with potential therapeutic values that may validate many of the therapeutic uses of the plant in traditional medicine. In particular, the leaf of *A. muricata* has garnered significant attention for its therapeutic value due to its high content of acetogenins, alkaloids, and phenolic compounds, which are associated with various biological functions, including antimicrobial, anti-inflammatory, and anticancer activities (Moghadamtousi *et al*., 2021).

Enterococcus (E.) faecalis, a gram-positive bacterium, has gained notable attention in recent years due to its increasing antibiotic-resistance profile and role as an opportunistic pathogen, particularly in healthcare settings. As a common cause of hospital-acquired bacteremia, endocarditis, wounds and urinary tract infections, *E. faecalis* has shown an alarming trend of resistance to several antibiotics, including those frequently used for treatment of drug resistant infections (Gajdács *et al*., 2020). Given the rising prevalence of antibiotic-resistant strains of this pathogen, and the urgent need for alternative treatment options, this study investigated the antimicrobial effects of *A. muricata* leaves extracts on urogenital isolates of *E. faecalis* from patients attending a tertiary hospital in Calabar, Nigeria.

MATERIALS AND METHODS

Collection and identification of *A. muricata* plant leaves

Annona muricata plant leaves (Voucher number: Bot/Herb/UCC 314), identified by Dr. Effa, Effa A., were collected from the Department of Plant and Ecological Science botanical garden in University of Calabar, Calabar, Nigeria. The plant leaves were washed and air-dried under shade to prevent degradation of its bioactive compounds.

Preparation of the extracts

The dried leaves of *A. muricata* were ground into a fine powder using a mechanical blender. The powdered plant materials were then subjected to successive ethanol and aqueous extraction using the Soxhlet extraction method as described by Chaaradi *et al*., 2020. The powdered plant material was placed in a thimble, and the solvent was continuously cycled through the sample to maximize the yield of the crude extract.

Collection of bacterial isolates and preparation of inoculum

A total of 20 urogenital isolates of *E. faecalis*, previously identified using VITEK 2 Compact System® (bioMérieux, Marcy L'Etoile, France) in the UCTH Microbiology Laboratory- Calabar, was used for the study. The inoculum of each isolate was prepared from fresh pure cultures on Mueller Hinton agar and standardized according to CLSI guidelines (CLSI, 2018). The density of the bacterial suspension was adjusted to match the opacity of 0.5 McFarland standard, corresponding to approximately 1.5 × 10^8 colony-forming units per milliliter (CFU/mL) (Wiegand *et al*., 2008). The standardized inoculant was used within an hour after preparation to ensure viability and consistency of the results.

Antibacterial susceptibility testing of *E. faecalis* isolates to *A. muricata* leaves extracts

The antibacterial effect of *A. muricata* leaves extract on *E. faecalis* isolates was evaluated using the agar-well diffusion method. The standardized inoculum of the test organism was streaked on freshly prepared Mueller Hinton agar, and holes measuring 6mm in diameter were made on the inoculated agar plates using a sterile cork-borer; aliquots (0.5 ml) of each dilution of the extract (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml) were carefully dispensed into the respective agar wells. Meropenem (10 µg) disc placed at center of the plate and 0.5% ethanol in one of the wells were used as positive and negative controls, respectively. The plates were kept on the bench for 30 minutes to facilitate diffusion of the extracts into the agar before incubation at 37^0C for 24 hours. Following overnight incubation, the agar plates were examined for bacterial growth and zones of inhibition around the wells measured in millimeters (mm).

Determination of minimum inhibitory concentration (MIC)

Two-fold serial dilutions of the extracts were prepared by adding 1 ml of the extracts stock solution (400 mg/ml) to 1 ml of freshly prepared Mueller Hinton broth in sterile glass test tubes (13x100 mm), to produce final concentrations of 200, 100, 50, 25, and 12.5 milligrams per milliliters (mg/ml).

Using a sterile 2ml syringe and needle, 1ml of the extract (200 mg/ml) was added to the second tube and the contents agitated on a Vortex mixer. 1 ml of the solution in the second tube was transferred to the third tube, and the process continued through the next to the last tube from which 1 ml was removed and discarded. No extract was added to one tube which served as a negative growth control, while Meropenem (10 µg/ml) was used as positive control. An equal volume of a fixed bacterial culture was added to the tubes and incubated at 37°C for 24 hours. The tubes were examined for turbidity following incubation. The lowest concentration that showed no visible growth (turbidity) was noted and recorded as the MIC value (Nwinyi *et al*., 2008).

Antibiotic susceptibility testing of the isolates

Antibiotic susceptibility testing of the *E. faecalis* isolates was carried out using Kirby-Bauer disc diffusion method, as described by Umoh *et al*.

(2024). Even spreads of the isolates were made on Mueller Hinton agar plates using sterile swab sticks followed by aseptic placement of antibiotics discs. The antibiotics discs used included Ciprofloxacin (5 µg), Levofloxacin (5 µg), Meroponem (10 µg), Cefoxitin (30 µg) and Gentamicin (10 µg). The plates were incubated at 37^{o}C for 24 hours, and after which examined for zones of inhibition. The zones edges were read against a dark background illuminated with reflected light, with each zone diameter measured using a ruler and interpreted according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2018).

Data analysis

The data obtained from this study were analyzed using Statistical Package for Social Sciences (SPSS) version 25.0 software. The susceptibility of *E. faecalis* isolates to different concentrations of *A. muricata* leaf extracts and conventional antibiotics was expressed in percentages. Minimum Inhibitory Concentration (MIC) values were reported in milligrams per milliliter (mg/ml). Chi-square test was used to evaluate the statistical significance of the extracts activity on the isolates in relation to extract-concentrations. The diameter of inhibition zones were measured in millimeters and presented as mean ± standard deviation. A one-way Analysis of Variance (ANOVA) was used for comparing zones of inhibition at different concentrations of the extracts. Probability values less than or equal to 0.05 were considered significant.

RESULTS

Antibacterial activity of ethanol extract of *A. muricata* against *E. faecalis* isolates

Antibacterial activity of the ethanol extract of *Annona muricata* leaves on isolates of *E. faecalis* is shown in Table 1.

Out of 20 *E. faecalis* isolates tested, 12 (60%), 10 (55%), and 6 (30%) were susceptible to *A. muricata* leaves extract at concentrations of 200 mg/ml, 100 mg/ml, and 50 mg/ml, respectively. In contrast, there was no inhibition of the isolates at lower concentrations of the extract, 25 mg/ml and 12.5 mg/ml. The antibacterial effects of the extract on the isolates was significantly associated with the extract's concentrations (p = 0.0032).

Antibacterial activity of aqueous extract of *A. muricata* against *E. faecalis* isolates

Table 2 shows the antibacterial activity of the aqueous extract of *Annona muricata* leaves against *E. faecalis* isolates.

Out of 20 *E. faecalis* isolates tested, 10 (50%), 7 (35%), and 5 (25%) were susceptible to *A. muricata* leaves extract at concentrations of 200 mg/ml, 100 mg/ml, and 50 mg/ml, respectively. In contrast, there was no inhibition of the isolates at lower concentrations of the extract, 25 mg/ml and 12.5 mg/ml. The antibacterial potency of the extract for the isolates was significantly associated with its concentrations (p = 0.0032).

Table1: Antibacterial activity of ethanol extract of *A. muricata* against *E. faecalis*

Concentration of Extract (mg/ml)	No. (%) of susceptible isolates (n=20)	p-value
200	12 (60)	
100	10 (50)	
50	6 (30)	
25	0 (0)	
12.5	0 (0)	0.0032*

*significant at P≤0.05

Table 2: Antibacterial activity of aqueous extract of *A. muricata* on *E. faecalis* isolates

Concentration of Extract (mg/ml)	No. (%) of susceptible isolates (n=20)	p-value
200	11 (55)	
100	7 (35)	
50	5 (25)	
25	0 (0)	
12.5	0 (0)	0.0032*

*significant at P≤0.05

Antibacterial potency of *A. muricata* leaf extracts against *E. faecalis* isolates

The inhibitory potency of *A. muricata* leaf extracts against *E. faecalis* isolates is presented in Table 3.

The ethanol extract showed a higher antibacterial potency with a mean inhibition-zone diameter of 16.5 ± 2.3 mm at concentration of 200 mg/ml, compared to that of aqueous extract with a mean zone of inhibition of 15.0 ± 1.0 mm at the same concentration. Similarly, at the MIC of 50mg/ml, the ethanol extract produced a higher inhibitory potency with a mean zone diameter of 10.2 ± 1.0 mm, compared to 9.8 ± 1.0 mm for the aqueous extract. The size of inhibition zones correlated significantly with the concentrations of the extracts (p= 0.012)

Minimum Inhibitory Concentration of *A. muricata* Leaf extract against *E. faecalis*

Assay results showed that the MIC of the ethanol and aqueous extracts of *A. muricata* leaves was 50 mg/ml for *E. faecalis* isolates (Table 4).

Antibiotic susceptibility pattern of *E. faecalis* isolates

Table 5 shows the antibiotic susceptibility pattern of *E. faecalis* isolates to some commonly used conventional antibiotics.

The susceptibility of *E. faecalis* isolates to some conventional antibiotics, including Levofloxacin, Ciprofloxacin, Meropenem, Cefoxitin, and Gentamicin, was 90%, 80%, 65%, 60%, and 55%, respectively.

Table 3: Inhibitory potency of *A. muricata* leaf extracts on *E. faecalis* isolates (n=20)

Extracts	Concentration (mg/ml)	Mean -Zone of inhibition diameter (mm)	F-value	p-value
Ethanol	200	16.5 ± 2.3	178.6	0.012*
	100	13.8 ± 1.9		
	50	10.2 ± 1.0		
	25	0		
	12.5	0		
Aqueous	200	15.0 ± 1.0		
	100	12.2 ± 1.7		
	50	9.8 ± 1.0		
	25	0		
	12.5	0		

Data presented as mean ± standard deviation
*Significant at p<0.05 (One-way ANOVA)

Table 4: Minimum Inhibitory Concentration of *A. muricata* Leaf extract against *E. faecalis*

Extracts	MIC
Ethanol	50 mg/ml
Aqueous	50 mg/ml

Table 5: Antibiotics susceptibility pattern of *E. faecalis* isolates (n=20)

	No. of isolates (%)				
	*CIP	LEV	CN	MEM	FOX
Sensitive	18 (90)	16 (80)	11 (55)	13 (65)	12 (60)
Resistant	2 (10)	4 (20)	9 (45)	7 (35)	8 (40)

*CIP- Ciprofloxacin (5µg), LEV- Levofloxacin (5µg), CN- Gentamicin (10µg), MEM- Meropenem (10µg), FOX- Cefoxitin (30µg).

DISCUSSION

The antimicrobial potentials of *A. muricata* extracts have been evaluated with promising results in many settings (Nguyen *et al*., 2022; Silva *et al*., 2023). The present study demonstrated the antibacterial activity of ethanol and aqueous extracts of *A. muricata* leaves on urogenital isolates of *E. faecalis*. A concentration-dependent antibacterial effect was found for both extracts against the isolates, in concordance with previous reports of similar studies (Solomon-Wisdom *et al*., 2014; Jemikalajah *et al*., 2021; Silva *et al*., 2023). The ethanol extract showed a slightly higher antibacterial efficacy, with 60% of the isolates susceptible at 200 mg/ml concentration compared to 50% for the aqueous extract. The difference in activity, though not weighty, may underscore the higher efficacy of ethanol as a solvent for extraction of the bioactive compounds with antibacterial properties in the plant leaves; compared with water, ethanol has been found to solubilize a wider range of compounds, including volatile oils and other lipophilic compounds that may have antibacterial properties (Rai and Kumar, 2013; Solomon-Wisdom *et al*., 2014).

The MIC value of 50 mg/ml found for both extracts of *A. muricata* leaves may indicate modest antibacterial potency against *E. faecalis* isolates. This concentration value was higher than those reported for other plant extracts against *E. faecalis* (Palombo, 2011). The high MIC values of the extracts for *E. faecalis* in this study may be due to presence of interfering substances that reduce the potency of bioactive or antimicrobial compounds in the crude extracts. Curiously, the finding of equal MIC values for both aqueous and ethanol extracts, in spite of the higher antibacterial effect of the latter in this study, may underscore a rich phytochemical profile of *A. muricata* leaves consisting of polar and non-polar compounds with antimicrobial properties. Phytochemicals such as alkaloids, flavonoids, tannins, steroids, saponins, oacetogenins, and phenolic compounds that act as antimicrobials have been associated with the plant leaves (Moghadamtousi *et al.,* 2015; Jemikalajah *et al*., 2021).

The antibacterial activity of *A. muricata* leaf extracts against *E. faecalis* isolates in this study was comparatively lower than that of some conventional antibiotics, including Ciprofloxacin (90%), Levofloxacin (80%), and Meropenem (65%). However, the susceptibility of the *E. faecalis* isolates to Cefoxitin (60%) and Gentamicin (55%) corresponded to that of the ethanol and aqueous extracts, respectively. These findings, however, is in contrast with a report of superior antibacterial activity of *A. muricata* leaves extracts, compared to some conventional antibiotics, against some multidrug-resistant bacterial strains (Pinto *et al.*, 2017).

CONCLUSION

This study found a modest antibacterial activity for *A. muricata* leaves extracts against urogenital isolates of *E. faecalis,* with a high MIC value of 50 mg/ml for both the ethanol and aqueous extracts. Although both extracts exhibited a concentration-dependent activity with equal MIC values, the ethanol extract produced a higher antibacterial potency against the study isolates. The efficacy of *A. muricata* extracts against *E. faecalis* isolates was lower than that of some commonly used antibiotics in this study. Identification, isolation and purification of the specific antimicrobial compounds present in the crude extracts would likely optimize its potency against bacterial pathogens, particularly multidrug resistant strains.

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