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Comparative Evaluation of the Effect of *Annona muricata* (Graviola) Leaves Extracts and Cefoxitin on *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors UEG, CIM, ENM and UOE designed the study. All authors did sample collection, Laboratory and Data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Resistance of *Staphylococcus aureus* to beta- lactam drugs most especially cefoxitin is a known global problem. Current researches are geared toward evaluating the antimicrobial properties of medicinal plants against these organisms. This study was aimed at comparatively evaluating the antimicrobial effects of cefoxitin and *Anonna muricata* (Graviola) against *Staphylococcus aureus* strains. A total of ten *Staphylococcus aureus* strains obtained from the Microbiology Department of General Hospital were subjected to susceptibility testing against cefoxitin and *Anonna muricata* extracts (both aqueous and ethanol), respectively using standard microbiology techniques. Out of the 10 isolates tested, 4 (40%) showed resistance to cefoxitin, 2 (20%) were intermediate while 4 (40%) showed susceptibility. However, 8 (80%) out of 10 isolates showed susceptibility to

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Annona muricata ethanol extract while 2 (20%) were resistance. In addition, 9 (90%) out of the 10 isolates showed resistance to *Anonna muricata* aqueous extracts. This study reveals that the isolates of *Staphylococcus aureus* were significantly more susceptible to alcohol extract of *A. muricata* than to cefoxitin consistently at concentration of 500-800 mg. There is a need for further studies aimed at evaluating its potentials against other isolates.

Keywords: Cefoxitin; graviola; antimicrobial resistance; Staphylococci.

1. INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that belongs to the family Staphylococcace a that has been observed as one of the most common pathogens of human and a leading cause of hospital acquired infections [1-3]. Besides being a commensal on the skin, glands and mucous membranes particularly in the nose of healthy individuals, this organism has been implicated in a host of hospital acquired infections leading to considerable morbidity and mortality [3-4]. These range from mild skin and soft tissue infections to life threatening sepsis, pneumonia, osteomyelitis, endocarditis as well as toxin mediated syndromes and food poisoning [5-7].

Prior to the introduction of penicillin for the treatment of *S.aureus* infections in the 1940s, the mortality rate of individuals with staphylococcal infections was about 80% [8]. Sadly, by 1960, about 80% of all *S. aureus* strains were found to be resistant to penicillin [9], heralding the introduction of cefoxitin in 1959 [10]. In 1961, cefoxitin-resistant hospital acquired *S. aureus* were reported [11].

In Nigeria, due to poor hospital hygiene practices, *S. aureus* strains most especially those associated with nosocomial infections have been reported to have developed resistance to antibiotics often used against them [3,12]. Resistance of *S. aureus*to most antibiotics introduced into general clinical practices has been reported thus, necessitating the need for alternative forms of therapy. One of which is the use of ethnomedicinal plants including (Graviola) for treatment of Staphylococcal diseases [13].

Some studies have extensively enumerated the use of Graviola extract as an alternative to antibiotics in patient management [13,14]. Graviola plant (*Annona muricata*) belongs to the family *Annonaeceae* and has a wide spread of tropical distribution. Studies have shown that the barks, fruits, leaves and seeds of Graviolaabound withover 100 acetogenins [15]. These compounds display some interesting biological and pharmacological activities such as antimicrobial, cytotoxic, anti-parasitic and pesticidal activities [13,15,16]. A few studies have shown thatGraviolaextracts have antibacterial activity against pathogens including *S. aureus.* This study was aimed at evaluating the antimicrobial activity of cefoxitinand extracts of *Annona muricata* against different *S. aureus strains*.

2. MATERIALS AND METHODS

2.1 Collection of Isolates and Identification

A total of 10 *Staphylococcus aureus* strains isolated from various clinical specimens from the Microbiology unit of General Hospital were used in this study. The isolates were appropriately preserved and transported immediately to the microbiology laboratory of the University of Calabar for further analysis. They were then inoculated into nutrient and mannitol salt agars, respectively. The plates were then incubated at 37°C for 24 hrs after which the isolates were then identified using standard microbiological technique previously described [17].

2.2 Collection and Preparation of Graviolaleaves

This was carried out as described by Ebana et al. [18] and Edet et al. [19]. Fresh leaves of *Graviola* were collected from behind the main library of the University of Calabar (4.9738° N, 8.3410° E) and were identified at the Department of Botany, University of Calabar. Following identification, the leaves were then rinsed with clean water, spread in a clean tray and allowed to air dry. The dried leaves were then heated in an electric oven to 60℃ for 2 hourrs. The leaves were then pulverized into powder and stored in sterile wide mouthed bottles until required for use.

2.3 Aqueous Extract Preparation

The modified method of Ebana et al. [18] and Edet et al. [19] were employed in carrying the aqueous extraction of the pulverized leaves. Exactly 50 g of the leaves were then dissolved in 1000 ml of sterile distilled water with continuous stirring for about 4 hours with heating at 40℃ in a water bath. After cooling, the extract was filtered by using Whatman no. 1 filter paper. The filtrate was collected, frozen in ice cube container. The frozen ice cube was freeze-dried (lyophilized) to obtained concentrated aqueous extract in powder form.

2.4 Ethanol Extracts Preparation

This was done as described by Ebana et al. [18] but with some modifications. Briefly, the fresh leaves of Graviola plant were mixed and macerated with 90% ethanol by dissolving 50 g of the pulverized leaves into 1000 ml of ethanol. The container was then wrapped with aluminium foil and kept for 7 days. The extract was filtered through Whatman No. 1 filter paper and then followed by evaporating the solvent using rotor evaporation (Rotary Evaporator, BUCHI Switzerland) and to obtain concentrated slurry extract. The crude extract was then kept at 4℃ in sterile universal bottles until required for use.

2.5 Sensitivity Test with Cefoxitin

The inoculum was prepared by emulsifying three to four discrete colonies of each test isolate in a sterile test tube containing peptone water and incubated for 30 minutes. The suspension was adjusted to match with 0.5 Mc Farland turbidity standard after which the peptone water isolate suspension was poured into a freshly prepared nutrient agar plate and swirled gently to cover the surface of the agar. Then, the antibiotic disc (cefoxitin) was placed aseptically on the surface of the inoculated plate using a sterile forceps and pressed lightly to ensure contact with the agar surface. The plate was incubated at 37℃ for 24hours. Examined zones of inhibition were compared with standard provided by Clinical Laboratory Standard Institute (CLSI) [20]. These procedures werethen repeatedfor all the test isolates.

2.6 Sensitivity Test with Graviola Extracts

Antimicrobial activity of the aqueous and ethanol extracts of the leaves was assayed using the paper disc diffusion method [20]. Discs of 5mm diameter were made from Whatman filter paper no. 1 using a paper puncher and sterilized in the oven 100℃ for 30 min. Various concentrations (mg/ml) (800, 500, 250, 125 and 62.5) were prepared after which 4 sterile discs each were immersed into each test tube containing the extracts respectively and allowed to stand for 10 minutes. Then, the discs were removed and dried in an oven at 40℃ for 15 minutes. Finally, discs were carefully and firmly placed on the plates containing freshly prepared nutrient agar lawned previously with the test organisms respectively. The plates were then incubated at 37℃ for 24 hrs, examined and zones of inhibition measured. For both extracts, gentamycin (10 µg) was used as control on all the test isolates.

2.7 Determination of MIC and MBC of Cefoxitin

This was done as previously reported [20]. Briefly, two to three colonies of the isolate were inoculated into 5ml of sterile peptone broth and incubated for 30 minutes. Exactly 30 µg of cefoxitin was used to prepare 7.50, 3.75, 1.875, 0.94, 0.47 and 0.23 µg/ml. One tube containing distilled water was kept as the control. Then, 0.5 ml of the inoculum was introduced into each of the test tubes after which the tubes were incubated at 37℃ for 24 hrs. After incubation, the tubes were observed for growth. This procedure was carried out on all test isolates. The MBC was determined by sub-culturing tubes which showed no growth (turbidity) during the MIC. A loopful from each test tube was sub-cultured into plates containing freshly prepared nutrient agar and incubated at 37ºC for 24 hours. The least concentration in the MIC test which showed no growth in the sub-culture plate was recorded as MBC. This was also repeated for all the test isolates in the study.

2.8 Determination of MIC and MBC of Graviola Extracts

Two to four colonies of the test isolate was inoculated into 10 ml of sterile nutrient broth and incubated for 30 minutes. Various concentrations (mg/ml) of Graviola extract were prepared ranging from 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, 50, 25 to 12.50. Then, 0.5 ml of each of the test organisms were introduced into the diluted extracts respectively and incubated at 37℃ for 24 hrs. After incubation,

the tubes were observed for turbidity.MBC procedure for cefoxitin was then repeated [20].

3. RESULTS

Table 1 shows the result of the sensitivity test carried out on S. *aureus* isolates against cefoxitin. Out of the 10 isolates that were subjected to testing, 4 (40%) isolates were susceptible, 2 (20%) isolates were intermediate while 4 (40%) isolates were resistant to cefoxitin. Table 2 and 3 show the result of the sensitivity test carried out on test isolates against Graviola extracts. Out of 10 isolates that were subjected to the ethanol extract, 7 (70%) were susceptible while 3(30%) isolates were resistant irrespective of the concentration that was used. However, the aqueous extract gave a rather different result. Only 1 (10%) was intermediate in sensitivity while the rest 9 (90%) were resistant. Compared to cefoxtin and aqueous extract, the highest zone of inhibition of 35 mm was observed in the ethanolic extract (800 mg/ml). Table 3 shows the result of the MIC and MBC of cefoxitin and Graviola (ethanol and aqueous) extracts. The results showed that cefoxitin had the highest MIC and the lowest MBC of 470 and 230 mg/ml, respectively. However, that of the ethanolic extract was 200 and 250 mg/ml, respectively.

4. DISCUSSION

Antibiotics susceptibility pattern of *Staphylococcus aureus* isolates showed that 4(40%) were resistant, 2(20%) were intermediate and 4(40%) were sensitive. Studies have shown that the high level of resistance usually observed with penicillin is as result of the fact that that this organism produces extracellular enzyme (beta-lactamase) that destroy the beta-lactam ring thus, rendering the drugs ineffective [10-13,21]. However, different resistant patterns of *S. aureus* strains against commonly used antibiotics were observed in this study. The high susceptibility to Ciprofloxacin makes it a better choice that can be used often in combination with Gentamicin or Rifampicin in the treatment of infection due to multi-resistance *Staphylococcus aureus*. This assertion is consistent with that observed by [22]. The high resistance recorded against penicillin is worrisome because these drugs are often used in the treatment of different kinds of human diseases. The concern has been expressed by so many researchers including Otajevwo and Momoh, [23] and calls for urgent review of treatment protocols. The finding in these studies reaffirms the multi-drugs resistance nature of *Staphylococcus aureus* strains irrespective of the site they are isolated. The high percentage of multiple antibiotics resistance *S.aureus*strains in this study is consistent with report of Omoigberale et al. [22] and Otajevwo and Momoh [23].

Table 1. Sensitivity test of isolates to cefoxitin

Isolates	Zone of inhibition (mm)	Interpretation
1	15	I
2	17	I
3	11	R
4	20	S
5	13	R
6	14	R
7	16	R
8	22	S
9	24	S
10	30	S

Keys: I = intermediate, R= resistant, S = susceptible

Table 2. Sensitivity test of Graviolaaqueous and ethanolic extracts against test isolates

Isolates	EE							AE						
	800	500	250	125	62.5	C	I	800	500	250	125	62.5	C	R
1	35	33	24	20	19	12	S	13	12	12	12	10	12	R
2	32	29	27	25	20	11	S	12	10	10	10	9	11	R
3	25	22	21	19	19	12	S	14	12	12	10	8	12	R
4	11	13	10	9	8	10	R	11	13	13	10	9	10	R
5	14	11	11	9	9	12	R	14	11	11	11	9	12	R
6	23	20	20	19	18	11	S	13	11	11	11	9	11	R
7	28	25	25	23	20	10	S	14	12	12	10	9	10	R
8	30	30	23	20	19	11	S	12	12	12	11	11	11	R
9	32	30	20	19	19	12	S	14	14	14	12	10	12	R
10	13	11	10	9	8	11	R	17	17	17	16	15	11	I

EE= ethanolic extract, AE = Aqueous extract, I = intermediate, S = susceptible, R= resistant and C= Control (gentamycin, 10 μg)

Table 3. MIC and MBC of Cefoxitin and Graviola extract (mg/ml)

	MIC	MBC
Cefoxitin	470	230
Graviola (ethanol extract)	200	250
Graviola (aqueous extract)	350	400

This findings herald's the importance of prudent prescription of antibiotics by Physicians. Though, MRSA is believed to be found mostly in hospital settings, these strains of organisms have been observed to be increasing in the community settings globally [21]. Surveillance data on methicillin *Staphylococcus aureus* (MRSA) strains in most countries of sub-Saharan Africa are scarce thus implicating public health awareness of the organism. In this study, MRSA prevalence of 4 (40%) is considered to be high due to inadequate data on MRSA in the study area.

Unlike cefoxitin, Annonaceae, contain a wide variety of acetogenins and has a wide spectrum of antibiotic effects [13]. The bioactivity of aqueous base and ethanol base Graviolaextracts on *S.aureus* were determined and from the results obtained, it was observed that acetogenins inhibit the growth of *S. aureus*. Many phytochemicals have been found in Graviola [14]. These phytochemicals have been reported to possess antimicrobial activities. The increasing emergence of antibiotic resistance has channelled the interest of research towards medicinal plants in search of new and non-toxic drugs [18].

In this study, ethanol extract was found to be more effective in killing test isolates than the aqueous extract. This may be due to the fact that water contains a lot of organic and inorganic compounds which may or may not interact to inhibit their overall activities. In addition, the active ingredients from the plant materials are not easily extractable in water and thus, presentethanol as a better solvent in extracting constituents from leaves of *Annonamuricata* [24]. The poor activities of the aqueous extract against *Staphylococcus aureus*observed in this study is in agreement with an earlier study [24] which showed that aqueous extract of plants generally exhibit little or no antimicrobial activities against micro-organisms.

In addition, studies have confirmed that plant extracts are generally more effective against Gram positive organisms than Gram negative organisms [25]. This explains why our Gram positive isolates in this study were considerably susceptible to extracts. This susceptibility may be due to the fact that the studied organisms possess cell walls which serve as target sites for acetogenins unlike the gram negative organisms that lack a cell wall thus making the binding of most acetogenins difficult. Ethanol extract was found to produce highest zone of inhibition (35 mm). The MIC value of ethanol and aqueous extract in our study shows that MIC of *Annona muricata* against *Staphylococcus aureus* strains were 350 mg and 200 mg respectively placing that of cefoxitin in between. Thus, Graviola extract could be a promising therapeutic agent in the near future.

5. CONCLUSION

The findings in this study have shown that graviola extracts especially the ethanolic extract hold some potentials that could be exploited further. Furthermore, the high level of resistance to the test antibiotics reveals the need to review antibiotics prescription protocols of these antibiotics to *S. aureus*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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