

1 **Comparative evaluation of the effect of *Annonamuricata* (Graviola) leaves extracts and**  
2 **cefoxitin on *Staphylococcus aureus*.**

3 **ABSTRACT**

4 Resistance of *Staphylococcus aureus* to beta- lactam drugs most especially cefoxitin is a known  
5 global problem. Current researches are geared toward evaluating the antimicrobial properties of  
6 medicinal plants against these organisms. This study was aimed at comparatively evaluating the  
7 antimicrobial effects of cefoxitin and *Annonamuricata*(Graviola) against *Staphylococcus aureus*  
8 strains. A total of ten *Staphylococcus aureus* strains obtained from the Microbiology  
9 Department of General Hospital were subjected to susceptibility testing against cefoxitin and  
10 *Annonamuricata* extracts (both aqueous and ethanol), respectively using standard microbiology  
11 techniques. Out of the 10 isolates tested, 4 (40%) showed resistance to cefoxitin, 2 (20%) were  
12 intermediate while 4(40%) showed susceptibility. However, 8 (80%) out of 10 isolates showed  
13 susceptibility to *Annonamuricata* ethanol extract while 2 (20%) were resistance. In addition, 9  
14 (90%) out of the 10 isolates showed resistance to *Annonamuricata*aqueous extracts. This study  
15 reveals that the isolates of *Staphylococcus aureus* were significantly more susceptible to alcohol  
16 extract of *A.muricata* than to cefoxitin but at consistently at concentration of 500-800mg. There is  
17 a need for further studies aimed at evaluating its potentials against other isolates.

18 Key words: cefoxitin, Graviola, Antimicrobial resistance, *Staphylococci*;

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## 31 INTRODUCTION

32 *Staphylococcus aureus* is a gram-positive bacterium that belongs to the family  
33 Staphylococcaceae that has been observed as one of the most common pathogens of human and a  
34 leading cause of hospital acquired infections [1]-[3]. Besides being a commensal on the skin,  
35 glands and mucous membranes particularly in the nose of healthy individuals, this organism has  
36 been implicated in a host of hospital acquired infections leading to considerable morbidity and  
37 mortality [3]-[4]. These range from mild skin and soft tissue infections to life threatening sepsis,  
38 pneumonia, osteomyelitis, endocarditis as well as toxin mediated syndromes and food poisoning  
39 [5]-[7].

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

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

51 Some studies have extensively enumerated the use of *Graviola* extract as an alternative to  
52 antibiotics in patient management [13, 14]. *Graviola* plant (*Annonamuricata*) belongs to the  
53 family *Annonaceae* and has a wide spread of tropical distribution. Studies have shown that the  
54 barks, fruits, leaves and seeds of *graviola-Graviola* abound with over 100 acetogenins [15]. These  
55 compounds display some interesting biological and pharmacological activities such as  
56 antimicrobial, cytotoxic, anti-parasitic and pesticidal activities [13, 15, 16]. A few studies  
57 have shown that *graviola-Graviola* extracts have antibacterial activity against pathogens including  
58 *S.aureus*. This study was aimed at evaluating the antimicrobial activity of cefoxitin and extracts  
59 of *Annonamuricata* against different *S.aureus* strains.

## 60 MATERIALS AND METHODS

### 61 Collection of isolates and identification

62 A total of 10 *Staphylococcus aureus* strains isolated from various clinical specimens from the  
63 Microbiology unit of General Hospital were used in this study. The isolates were appropriately  
64 preserved and transported immediately to the microbiology laboratory of the University of  
65 Calabar for further analysis. They were then inoculated into nutrient and mannitol salt

66 | agars,respectively. The plates were then incubated at 37<sup>o</sup>C for 24hrs after which the isolates  
67 | were then identified using standard microbiological technique previously described [17].  
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#### 69 | **Collection and preparation of ~~graviola~~-Graviola leaves**

70 | This was carried out as described by Ebana et al[18] and Edet et al[19]. Fresh leaves of  
71 | *Graviola*were collected from behind the main library of the University of Calabar(4.9738° N,  
72 | 8.3410° E)and were identified at the Department of Botany, University of Calabar. Following  
73 | identification, the leaves were then rinsed with clean water,spread in a clean tray and allowed to  
74 | air dry. The dried leaves were then heated in an electric oven to 60°C for 2hourrs. The leaves  
75 | were then pulverized into powder and stored in sterile wide mouthed bottles until required for  
76 | use.

#### 77 | **Aqueous extract preparation**

78 | The modified method of Ebana et al [18] and Edet et al [19] were employed in carrying the  
79 | aqueous extraction of the pulverized leaves.Exactly 50g of the leaves were then dissolved in  
80 | 1000ml of sterile distilled water with continuous stirring for about 4 hours with heating at 40°C  
81 | in a water bath. After cooling, the extract was filtered by using Whatman no. 1 filter paper. The  
82 | filtrate was collected, frozen in ice cube container. The frozen ice cube was freeze-dried  
83 | (lyophilized) to obtained concentrated aqueous extract in powder form.

#### 84 | **Ethanol extracts Preparation**

85 | This was done as described by Ebana et al [18] but with some modifications. Briefly, the fresh  
86 | leaves of ~~graviola~~-Graviolaplantwere mixed and macerated with 90% ethanol by dissolving 50g  
87 | of the pulverized leaves into 1000ml of ethanol. The container was then wrapped with  
88 | aluminium foil and kept for 7 days. The extract was filtered through Whatman No. 1 filter paper  
89 | and then followed by evaporating the solvent using rotor evaporation (Rotary Evaporator,  
90 | BUCHI Switzerland) and to obtain concentrated slurry extract. The crude extract was then kept  
91 | at 4<sup>o</sup>C in sterile universal bottles until required for use.

#### 92 | **Sensitivity test with cefoxitin**

93 | The inoculum was prepared by emulsifying three to four discrete colonies of each test isolate in a  
94 | sterile test tube containing peptone water and incubated for 30 minutes. The suspension was  
95 | adjusted to match with 0.5 McFarland turbidity standard after which the peptone water isolate  
96 | suspension was poured into a freshly prepared nutrient agar plate and swirled gently to cover the  
97 | surface of the agar. Then, the antibiotic disc (cefloxitin) was placed aseptically on the surface of  
98 | the inoculated plate using a sterile forceps and pressed lightly to ensure contact with the agar  
99 | surface. The plate was incubated at 37<sup>o</sup>C for 24hours.Examined zones of inhibitionwere

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100 compared with standard provided by Clinical Laboratory Standard Institute (CLSI) [20].These  
101 procedures werethen repeatedfor all the test isolates.

#### 102 | Sensitivity test with ~~graviola~~-Graviolaextracts

103 Antimicrobial activity of the aqueous and ethanol extracts of the leaves was assayed using the  
104 paper disc diffusion method [20]. Discs of 5mm diameter were made from Whatman filter paper  
105 no. 1 using a paper puncher and sterilized in the oven 100<sup>0</sup>C for 30min. Various concentrations  
106 (mg/ml) (800, 500, 250, 125 and 62.5)were prepared after which 4 sterile discs each were  
107 immersed into each test tube containing the extracts respectively and allowed to stand for 10  
108 minutes. Then, the discs were removed and dried in an oven at 40<sup>0</sup>C for 15 minutes. Finally,  
109 discs were carefully and firmly placed on the plates containing freshly prepared nutrient agar  
110 lawned previously with the test organisms respectively. The plates were then incubated at 37<sup>0</sup> C  
111 for 24hrs, examined and zones of inhibition measured. For both extracts, gentamycin (10□g) was  
112 used as control on all the test isolates.

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#### 114 | Determination of MIC and MBC of cefoxitin

115 This was done as previously reported [20]. Briefly, two to three colonies of the isolate were  
116 inoculated into 5ml of sterile peptone broth and incubated for 30minutes. Exactly 30μg of  
117 cefoxitin was used to prepare 7.50, 3.75, 1.875, 0.94, 0.47 and 0.23 μg/ml. One tube containing  
118 distilled water was kept as the control. Then, 0.5ml of the inoculum was introduced into each of  
119 the test tubes after which the tubes were incubated at 37<sup>0</sup>C for 24hrs. After incubation, the tubes  
120 | were observed for growth. This procedure was carried out on all test isolates.—The MBC was  
121 determined by sub-culturing tubes which showed no growth (turbidity) during the MIC. A  
122 loopful from each test tube was sub-cultured into plates containing freshly prepared nutrient agar  
123 | and incubated at 37<sup>-0</sup>C for 24hours. The least concentration in the MIC test which showed no  
124 growth in the sub-culture plate was recorded as MBC. This was also repeated for all the test  
125 isolates in the study.

#### 126 | Determination of MIC and MBC of ~~graviola~~-Graviolaextracts

127 Two to four colonies of the test isolate was inoculated into 10ml of sterile nutrient broth and  
128 incubated for 30minutes. Various concentrations (mg/ml) of Graviola extract were prepared  
129 ranging from 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, 50, 25 to 12.50. Then, 0.5ml  
130 of each of the test organisms were introduced into the diluted extracts respectively and incubated  
131 at 37<sup>0</sup> C for 24 hrs. After incubation, the tubes were observed for turbidity.MBC procedure for  
132 cefoxitin was then repeated [20].

#### 133 | 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 cefoxitin and aqueous extract, the highest zone of inhibition of 35mm was observed in the ethanolic extract (800mg/ml). Table 3 shows the result of the MIC and MBC of cefoxitin and ~~graviola~~ 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.

**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 Graviola aqueous 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 g)

Comment [MS1]: ?????

**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 |

# 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][23].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 [21]. 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, [22] 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 Omoigberaleet *al.*, [21] and Otajevwo and Momoh[22].

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 [23]. 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

178 prevalence of 4 (40%) is considered to be high due to inadequate data on MRSA in the study  
179 area.

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

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

195 In addition, studies have confirmed that plant extracts are generally more effective against  
196 Gram positive organisms than Gram negative organisms [25]. This explains why our Gram  
197 positive isolates in this study were considerably susceptible to extracts. This susceptibility may  
198 be due to the fact that the studied organisms possess cell walls which serve as target sites for  
199 acetogenins unlike the gram negative organisms that lack a cell wall thus making the binding of  
200 most acetogenins difficult. Ethanol extract was found to produce highest zone of inhibition  
201 (35mm). The MIC value of ethanol and aqueous extract in our study shows that MIC of  
202 *Annonamuricata* against *Staphylococcus aureus* strains were 350mg and 200mg respectively  
203 placing that of cefoxitin in between. Thus, ~~graviola~~ ~~Graviola~~ extract could be a promising  
204 therapeutic agent in the near future.

## 205 Conclusion

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

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