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# ANTIBIOGRAM OF *CITRUS MAXIMA* AGAINST MULTIDRUG-RESISTANT *STAPHYLOCOCCUS AUREUS, ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

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**ABSTRACT**

Pathogenic microorganisms that have developed multi-resistance to major classes of antibiotics have become worrisome. The antibacterial activities of the ethyl acetate extracts of citrus maxima obtained via the agar well diffusion method against multidrug -resistant *Pseudomonas aeruginosa*, Escherichia coli and *Staphylococcus aureus* from clinical samples were examined. The inhibition zone diameter (IZD) of the plant extract against *Pseudomonas aeruginosa* was greatest at 50 mg/ml and 12.5 mg/ml, resulting in an inhibition zone diameter of 12 mm, followed by that of *Staphylococcus aureus* at 100 mg/ml and 25 mg/ml at 8 mm and that of *Escherichia coli* at 100 mg/ml and 50 mg/ml. Ciprofloxacin (positive control) was effective at all concentrations, but no activity was detected in the sterile distilled water used (negative control). The minimum inhibitory concentration and maximum bactericidal concentration results revealed that *Pseudomonas aeruginosa* was highly susceptible to the plant extract. This result confirmed the efficacy of the plant in inhibiting bacterial growth.

**KEYWORDS:** *Citrus maxima*, Multidrug-resistant bacteria, Agar well diffusion, Minimum inhibitory concentration (MIC).

## INTRODUCTION

Plants have been utilized as source of herbal medicines since ancient times and the presence of secondary metabolites in plants have implicated them for many therapeutic applications. The plants also act as a source of inspiration for development of novel drug compounds, as plant derived medicines have made large contributions to human health and diseases (Anjun &Mir., 2011). According to the World Health Organization, almost 80% of the world's population relies on traditional medicines for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body (Pawar *et al*., 2012).

Emergence of pathogenic microorganisms that are resistant or multi-resistant to major class of antibiotics has increased in recent years due to indiscriminate use of synthetic antimicrobial drugs(Cushnie & Lamb, 2005). In addition, high cost and adverse effects are commonly associated with popular synthetic antibiotics as a major burning global issue in treating infectious diseases (Bedaway 2010). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re- emerging infectious diseases. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections (Kumar *et al*., 2012). Antimicrobial of plant origin have enormous therapeutic potential (Firas& Hassan, 2008). They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Haja *et al*., 2014). The plant, Citrus maxima (Burm.) Merr. (Rutaceae), is commonly known as shaddock or pomelo. The plant is indigenous to tropical parts of Asia. The fruit and pulp are cited as anti-toxic, appetizer, cardiac stimulant and stomach tonic in ancient and medieval literature. Recently, leaves are found to exhibit anti tumour activity (Sen *et al*., 2011). Alcoholic extracts of the fruit also show antidiabetic and anti-hyperlipidemic potential (Mashkor, 2014; Fidrianny et *al*., 2016). The essential oil of the fruit shows in-vitro activity against *Staphylococcus aureus* and *Escherichia coli* (Oyedepo *et al*., 2012). Antibacterial activity of the unripe epicarp of Citrus maxima has not been evaluated so far. In this study, an attempt has been made to evaluate the antibacterial activity of the unripe epicarp of Citrus maxima against *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa.*

## MATERIALS AND METHODS

### Materials

**Media:** Muller-Hinton agar was used

### Instrument/Equipment

The following instrument/ equipment was used - swab stick, incubator, autoclave, Petri dish, Bunsen burner, weighing balance (Olympus USA).

### Sample collection

Unripe fresh fruits of Citrus maxima were collected from Aguogboriga, Ebonyi State. The fruits of the plant were also authenticated by a taxonomist in Faculty of science, Ebonyi State University (EBSU), Associate professor Kate Nnamani.

### Microorganisms

Pathogenic bacteria isolates of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was obtained from the department of microbiology Ebonyi State University (EBSU). The organisms were sub-cultured and stored in a semi solid medium until when needed.

### Preparation Of Culture Media

A 5.8g of Muller-Hinton agar was weighed out and dissolved in 120 ml of distilled water by heating over a Bunsen burner in a conical flask and autoclaved at $121^0$ c for 15mins. It was allowed to cool at $45^0$ c and 20ml each was aseptically poured into the Petri dish and allowed to gel. This was incubated for 18-24 hours at $37^0$ C.

### Inoculation Of Test plates

A three different sterile cotton swab stick each was dipped into the plates containing pathogenic isolates of Staphylococcus aureus, *Escherichia coli and Pseudomonas aeruginosa* and was rotated to collect a loop full of the inoculum. Subsequently, the dried surface of Muller-Hinton agar plate was inoculated by streaking two or more times, rotated the plate approximately $60^0$ each time to ensure an even distribution of inoculum and as a final step, the rim of the agar was equally swabbed.

### Preparation Of Herbal Extract

Fresh fruits of pomelo were collected, and the back was peeled off and was cut into different smaller sizes. The endocarp containing the fruit juice were carefully removed pounded into finely particles with the aid of ceramic mortar. Thereafter, 50g of the sample was weighed in a weighing balance and was dissolved in 400ml of 95% Ethyl acetate contained in the round bottom conical flask. The conical flask was corked and soaked for 24 hours. Subsequently, the solution was filtered using filter cloth. The plant residue was discarded. The filtrate was concentrated by allowing to air dry. The concentrated ethyl acetate extract was then diluted with 4ml of dimethyl sulphoxide (DMSO) solution before carrying out antibacterial activities and other microbiological screening

## ANTIBACTERIAL SUSCEPTIBILITY TEST

### Determination Of Minimum Inhibitory Concentration Of Ethyl acetate Epicarp Extract Of Citrus maxima

Varying concentration which includes 100ug/ml, 50ug/ml, 25ug/ml, 12.5ug/ml, 6.25ug/ml and 3.125ug/ml of ethyl acetate was prepared with sterile water. The surface of Muller-Hinton agar plates was streaked with the standardized inoculum of the test bacteria. Thereafter, a sterilized 6mm cork borer was used to bore 5 holes on the Muller-Hinton agar plates and four of the holes was filled with equal volume of the Citrus maxima extract that was diluted, the plates was incubated at $37^0$ C for 18-24 hours. The least concentration of the extracts that inhibits the growth of test organisms was taken as the minimum inhibitory concentration.

### Screening For Antibacterial Activity Of Ethyl Acetate Epicarp Extract Of Citrus maxima against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa.

Twenty milliliter of sterilized Muller-Hinton agar was poured aseptically into sterile petri-dish and allowed to solidify. The surface of the Muller-Hinton agar plates will be streaked with standardized inoculum of the test bacteria. Thereafter, a sterilized 6mm cork borer was used to bore 5 holes (1 hole in the middle which should contain ciprofloxacin as a positive control) on the Muller-Hinton agar plates and the remaining 4 holes was filled with equal volume of the respective plant extracts that was diluted with 0.5 dimethyl sulphoxide (DMSO). Sterile water was used as a negative control.

### Biochemical Tests

The following biochemical tests were used to reconfirm the isolates following the instructions of Cheesbrough, (2002).

### Catalase Test

This test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result. Staphylococcus spp and the Micrococcus spp are catalase positive. The Streptococcus and Enterococcus spp. are catalase negative.

### Citrate Utilization Test

Koser's citrate medium is selective to favors the isolation of organisms able to make use of citrate as sole source of carbon for growth. Citrate medium was inoculated with a straight wire that already contains a small amount of the isolate. It was then inoculated at 37°C for 5 days. A gradual change of color from green to bright blue (with turbidity showing growth) indicating a positive test (Chesbrough, 2002).

**Indole Test**

This test was used for the differentiation of coliforms and this depends on the production of indole by bacterium. About 5 ml of peptone water was dispensed into test tubes and inoculated with a loopful of broth culture of the bacteria under study and incubated for 5-7 days at 37°C. After incubation, 0.5 ml Kovac's indole reagent was added, shaken gently and then allowed to stand for 5 minutes. The development of a deep red coloration in the presence of indole which separates out in the alcohol layer indicated a positive test (Chesbrough, 2006).

**Methyl Red / Voges-Proskauer (MR/VP)**

This test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The stable production of enough acid to overcome the phosphate buffer will result in a pH of below 4.4. If the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red color will appear (first picture, tube on the left). If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized. The 2,3 butanediol fermentation pathway will ferment glucose and produce a 2,3 butanediol end product instead of organic acids. In order to test this pathway, an aliquot of the MR/VP culture is removed and naphthol and KOH are added. They are shaken together vigorously and set aside for about one hour until the results can be read. The Voges-Proskauer test detects the presence of acetoin, a precursor of 2,3 butanediol. If the culture is positive for acetoin, it will turn "brownish-red to pink". If the culture is negative for acetoin, it will turn "brownish-green to yellow". Note: A culture will usually only be positive for one pathway: either MR+ or VP+. Escherichia coli is MR+ and VP-. In contrast, Enterobacter aerogenes and Klebsiella pneumoniae are MR- and VP+. Pseudomonas aeruginosa is a glucose non fermenter and is thus MR- and VP-.

**3.8.5 Catalase Test**

This test was used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result. The sample on the right below is catalase positive. The *Staphylococcus* spp. and the Micrococcus spp. are catalase positive. The *Streptococcus* and *Enterococcus* spp. are catalase negative.

**3.9. Determination Of Inhibition Zone Diameter (IZD)**

A zone of inhibition (IZD) is the area on an agar plate where growth of bacteria is prevented in the presence of antibiotics or other antimicrobial compounds. The dimension of the zones can be measured as an indicator of the effectiveness of the antibiotics. The IZD was simply measured with a ruler in millimeter. This was done by locating a portion in the agar plate around the inoculated bacterium that has a clear zone and then subsequently placing the meter rule over it and record the value.

**RESULT AND DISCUSSION**

**Percentage yield of the plant extract**

The obtained after extraction of the herbal plant is as shown in Table 1 below.

**Table 1: Percentage yield.**

| S/no | Sample parts | Mass before extraction | Mass after extraction | Percentage yield (%) |
|---|---|---|---|---|
| 1. | UE | 50 | 33.4 | 66.8 |

UE=unripe epicarp

**Morphological, Biochemical and Physiological Characteristics of bacteria isolate**

The results obtained after re-characterization of the test isolate is as shown below in Table 2.

**Table 2: Morphological, Biochemical and Physiological Characteristics of bacteria isolate.**

| Morphological | | Biochemical tests | | | | | | | | Organisms |
|---|---|---|---|---|---|---|---|---|---|---|
| Shape | Color | Gram RXN | IN | CAT | CIT | TSI | VP | MR | SH | |
| Cocci | Yellow | + | - | + | + | - | - | - | - | *Staphylococcus aureus* |
| Rod | green | - | - | + | + | k/k | - | - | + | *Pseudomonas aeruginosa* |
| Rod | Pink | - | + | + | - | - | - | - | + | *Escherichia coli* |

**KEY**: IN = Indole test, CAT = Catalase test, CIT = Citrate test, TSI = Tryptone sugar hydrolysis, VP = Voges Proskauer test, MR = Methyl Red test, SH = Starch Hydrolysis, k/k = Alkaline/Alkaline

**Inhibition Zones diameter of the extracts of ethyl acetate at different concentration against selected microorganisms.**

In all the concentrations, *Pseudomonas aeruginosa* was found to exhibit the highest inhibition zone at both 50 mg/ml and 12.5 mg/ml followed by *Staphylococcus aureus* and then *Escherichia coli* at both 100 and 25 mg/ml.

**Table 3: Zones of inhibition as shown by ethyl acetate extract at different concentration against selected microorganisms.**

| Microorganisms | Concentration(mg/ml) /Zones of inhibitions (mm) | | | | | |
|---|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 | 3.125 |
| *Pseudomonas aeruginosa* | 6 | 12 | 8 | 12 | 8 | 6 |
| *Escherichia coli* | 4 | 4 | - | - | - | - |
| *Staphylococcus aureus* | 4 | - | 4 | 2 | - | - |

- = no inhibition

**MIC and MBC of ethyl acetate epicarp extract of Citrus maxima against Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus.**

From Table 4, it was observed that *Pseudomonas aeruginosa* have the least MIC(6.25 mg/ml) and MBC(12.5 mg/ml) followed by *Escherichia coli* (25.0mg/ml, 12.5mg/ml) and *Staphylococcus aureus* (50 mg/ml, 3.125 mg/ml).

**Table 4: MIC and MBC of ethyl acetate epicarp extract of Citrus maxima against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*.**

| Microorganisms | Concentrations EECM (mg/ml) | | | | | | MIC | MBC |
|---|---|---|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | | |
| *P. aeruginosa* | + | + | + | + | + | + | 6.25 | 12.5 |
| *Escherichia coli* | + | + | - | - | - | - | 25 | 12.5 |
| *S. aureus* | + | - | + | + | - | - | 50 | 3.125 |

+ Growth, - no growth.

## DISCUSSION AND CONCLUSION

### Discussion

All the isolates of Pseudomonas *aeruginosa, Escherichia coli* and *Staphylococcus aureus* were found to be susceptible to the ethyl acetate epicarp extract of Citrus maxima at (100mg/ml) concentrations. Both *Pseudomonas aeruginosa and Escherichia coli* were found to be susceptible at concentration (50mg/ml) whereas *Pseudomonas aeruginosa* and *Staphylococcus aureus* were susceptible at both concentration (25mg/ml and 12.5mg/ml). this result is in agreement with the findings of Sripana et al., (2011) states that the peel of the citrus fruit contains a higher percentage of antioxidants as compared to its pulp, as the purpose of the peel is to protect the antioxidants in the fruit from oxidation (Shiyananda & Muralidhara, 2013). Therefore, it is recommended to consume the fruit together with its peel.

Maximum zone of inhibition was shown by *Pseudomonas aeruginosa*. MIC value of the extract for *Peudomonas aeruginosa* (6.25mg/ml) was found to be lower than *Escherichia coli* (25mg/ml) while that of *Staphylococcus aureus* was found to be the greatest (50mg/ml) and also MBC value was found to be the same for both *Pseudomonas aeruginosa* and *Staphylococcus aureus* (3.125mg/ml) but was found to be different for both *Pseudomonas aeruginosa* and *Escherichia coli* (3.125mg/ml and 12.5mg/ml). This result is comparable to the work of Arumugam and Gunasekaran (2014). This is also in line with Mukundam & Swanamoni,(2013) as cited in Narayana et al., (2001) state that pomelo extracts have been revealed to have an antimicrobial activity against several bacteria such as *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Salmonella enteritidis and Escherichia coli.* Commonly used as a sweet delicacy, pomelo is overlooked as a source of prevention in microorganism proliferation (Ricardo et al., 2011; Prieto et al., 2016). A study by Prasad & Rajkumar, (2014) showed that citrus fruits exhibited antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureu*s. Further, the study in the King Mongkut's University of Technology North Bangkok stated that the effect of crude extract from pomelo peel can be used to inhibit Staphylococcus aureus (Paiwan & Linda, 2014).

Among the bacteria isolates used, *Pseudomonas aeruginosa* showed a higher sensitivity to the plant extract compared to *Escherichia coli* and *Staphylococcus aureus (*Potdar, 2011*)*. This signifies potent antibacterial activity of the extract because *Pseudomonas aeruginosa* is the most common gram-negative bacterium responsible for the nosocomial as well as community acquired infections. The development of multidrug resistant *Pseudomonas aeruginosa* is currently one of the greatest challenges to the effective management of infections (Anjum and Mir, 2010; Kundusen & Gupta, 2011).

## CONCLUSION

Based on the findings of the study, it was concluded that the pomelo peel extract showed positive results in inhibiting the growth of bacteria from the three selected microorganisms. The epicarp extract of pomelo has showcased its capability to completely inhibit the proliferation or spread of Escherichia coli and Pseudomonas aeruginosa. The extract exhibited a partial or slight inhibitory activity against Escherichia coli. The higher concentration showed a better antibacterial effect on *S. aureus* which implies more components of the pomelo peel are essential in order to increase the effectiveness of the antibacterial properties from the pomelo epicarp.

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