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RESISTANCE DEVELOPMENT IN MULTI-DRUG RESISTANT BACTERIA TO ANTIMICROBIALS FROM METHANOLIC EXTRACT OF THE STEM BARK OF *Artocarpus altilis*

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ABSTRACT

Antibiotics for many years were known to be effective against different strains of bacteria and other microbes. The discovery of antibiotics has led to a severe reduction in death and disease. However, the indiscriminate use of antibiotics resulted in the development of resistance and multiple resistances by bacteria hence, the search for alternative therapy for the treatment of infectious diseases making researchers shift their attention to plant antimicrobials. Nevertheless, the possibility of bacteria developing resistance to plant antimicrobials is not well studied. This study aims to evaluate the development of resistance in multi-drug resistant bacteria to antimicrobials from the methanol extract of the stem bark of *Artocarpus altilis*. The susceptibility of multi-drug resistant *Staphylococcus epidermis*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus saprophyticus*, *Proteus mirabilis* and *Pseudomonas aeruginosa* to methanol extract of *Artocarpus altilis* at different concentrations (160mg/l, 80mg/l, 40mg/l and 20mg/ml) was tested after which the bacteria were exposed to a sub-lethal concentration over some time. The result displayed that *Proteus mirabilis* showed no sensitivity at all concentrations after eighteen days of exposure. In comparison, the other bacterial test isolates only showed activity at 160mg/ml and 80mg/ml after 24 days of exposure except for *Bacillus cereus*, which showed activity at the lowest concentration (20mg/ml). This study demonstrated that exposure of bacteria to antimicrobial plant extract at a sub-lethal concentration could induce the development of resistance.

Contribution/Originality: The paper's primary contribution is finding that exposure of bacteria to antimicrobial plant extract at a sub-lethal concentration could induce the development of resistance of the bacteria to the antimicrobial plant extract.

1. INTRODUCTION

Ever since the discovery of antibiotics and their uses as chemotherapeutic agents, it was believed by the medical community that this would lead to the eradication of infectious diseases. It was once thought that diseases and disease agents, were controlled by antibiotics, but new forms are evolving that are resistant to Antibiotic therapies (Benjamin, Hungate, & Price, 2017). Antibiotic is a chemical made by one microorganism that could either kill or inhibit the growth of other microorganisms (Rebecca, Jangid, Shukla, & Dutta, 2019). They are the most active

chemotherapeutics among drugs; they exert their therapeutic effect by antagonizing the growth of bacteria (Salma & Rafik, 2015). Globally, in both the treatment and prophylaxis of people, animals and aquaculture millions of kilograms of antibiotics are used each year, thereby increasing the resistance problem by killing susceptible strains and selecting those that are resistant (Benjamin et al., 2017). The global emergence of multi antibiotics resistant bacterial strains is increasing, thereby resulting in the reduction of the effectiveness of current drugs, resulting in treatment failure of infections, thus becoming a major therapeutic problem (Anna & Braun, 2015). The occurrence of drug resistance in the community has extended the resistance problem beyond the limits of the hospital. Resistant strains can be traced from the hospital to the environment and vice versa, signifying that drug resistance is no longer localized (Anna & Braun, 2015).

Consequently, researchers are gradually turning their focus to herbal products, looking for new leads to produce better drugs against multi-drug resistant microbe strains (Srivastava, Chandra, Nautiyal, & Kalra, 2014). It has been scientifically proven that many plants or herbs contain bioactive compounds which has significantly led to the increase in request for herbal drugs in current times and is also alternatives to harmful synthetic drugs that cause side effects to a biological system and environment (Vadhana, Singh, Bharadwaj, & Singh, 2015). Several studies reported the use of herbal drugs, but there is little or no information on the possibility of the development of resistance by bacteria to plant antimicrobials (Ruiz, 2017). As a result, this study aims to evaluate the development of resistance in multi-drug resistant *Staphylococcus epidermis*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus saprophyticus*, *Proteus mirabilis* and *Pseudomonas aeruginosa* to antimicrobials from methanolic extract of the stem bark of *Artocarpus altilis*.

2. MATERIALS AND METHODS

2.1. Plant Material

The stem bark of *Artocarpus altilis* (Parkinson) Fosberg, growing in natural conditions, was collected from the University of Ibadan, Ibadan, Nigeria. The plant was identified and authenticated at the Herbarium Unit of Department of Botany, University of Ibadan, Ibadan, Nigeria.

2.2. Bacterial Cultures

The multi-drug resistant bacteria (*Staphylococcus epidermis*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) utilized in this study were obtained from the Microbiology Laboratory, Department of Microbiology, University of Ibadan, Ibadan Nigeria. Gram staining, morphological and biochemical tests were carried out to confirm the bacteria obtained before use.

2.3. Preparation of Crude Extract

The extraction procedure was carried out at the Pharmaceutical Chemistry Laboratory, Department of Pharmacy, University of Ibadan. The sample was transported to the laboratory, thoroughly washed under running tap water to remove debris and dust particles, and then air-dried for three weeks under room temperature until constant weight.

The stem bark was pulverized using mortar and pestle into a fine powder. One kilogram (1kg) of pulverized stem bark was poured into a well-labelled clean glass jar containing 1 litre of methanol and was left for 72 hours with intermittent stirring at 24 hours interval. After 72 hours, it was sieved using a muslin cloth and filtered with No. 1 Whatman filter paper. The concentration of the filtrate was done using a rotary evaporator at 40^{0}C. The extract was collected in an airtight bottle and refrigerated to preserve it until further use (Abalaka, Daniyan, Oyeleke, & Adeyemo, 2012).

2.4. Phytochemical Screening

Different phytochemicals, viz. saponins, tannins, cardiac glycosides, flavonoids, terpenoids, steroids, alkaloids, and anthraquinone were screened in the laboratory according to Sofowora standard method with little modification (Erum, Salim, & Lim, 2015; Sofowora, 1993).

2.5. Extract Concentration for Antibacterial Testing

A double fold dilution of the crude extract was done to obtain the required extract concentrations for antibacterial testing; the stock solution was prepared by dissolving sixteen (16) grams of the crude extract in 100ml dimethylsulfoxide (DMSO) to give a concentration of 160mg/ml. A second dilution was obtained by taking 5ml from the well-dissolved stock solution into 5ml of DMSO to give 80mg/ml. This dilution process was repeated to give a concentration of 20mg/ml, 10mg/ml and 5mg/ml (Pai-Wei, Yang, Yang, Su, & Chuang, 2015).

2.6. Antibacterial Activity Assay of the Artocarpus altilis by Agar Well Diffusion method

The agar well diffusion method was used. Overnight broth cultures of the microbial strains showing an absorbance value ranging between 0.129 – 0.134 at a wavelength of 625nm (i.e., equivalent to 0.5 McFarland of culture) were used for testing antimicrobial activity. Mueller–Hinton agar plates were swabbed using sterile cotton swabs with the adjusted broth culture of the respective bacteria strains. Five wells (6 mm in diameter) were prepared at equal distance in each of the plates using a sterile cork borer. Up to 100 µl (0.1 ml) of each concentration of the extract were respectively introduced into the wells using sterile automatic pipettes, with the stock solution in the centre well. It was allowed to diffuse at room temperature for 2hrs. The plates were incubated at 37°C for 24hrs. The solvent control used was DMSO, while ofloxacin disc (30µg) was used as the control antibiotic. Diameters of the inhibition zones were measured. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by the plant extract (Pai-Wei et al., 2015).

2.7. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Extract

MIC using the macro-broth dilution method with some modification was determined as the lowest concentration of the extracts allowing no visible growth (no turbidity) when compared with the control tubes. To 0.1ml of varying concentrations (5, 10, 20, 40, 80, and 160 mg/ml) of the plant extract (*Artocarpus altilis)* in test tubes, nutrient broth (9 ml) was added and then a loopful of the multi-drug resistant bacteria. A tube containing nutrient broth was only inoculated with the multi-drug bacteria to serve as the control. The culture tubes were then incubated overnight at 37°C. After incubation, the tubes were then examined for turbidity (an indication of microbial growth). The tube containing the lowest concentration of plant extracts showing no visible sign of growth was considered as the MIC. To determine the MBC of the plant extract (*Artocarpus altilis)*, from the nutrient broth incubated overnight, 1ml was collected from the tubes that showed no growth and inoculated onto sterile nutrient agar. The inoculated plates were then incubated for 24 hrs at 37°C. After incubation, the concentration that showed no visible growth was considered as the MBC (Akinyemi, Oluwa, & Omomigbehin, 2006; Pai-Wei et al., 2015).

2.8. Resistance Analysis of the Methanol Extract of Artocarpus altilis

The multi-drug resistant bacteria were sub-cultured in nutrient broth containing a concentration lower than the Minimum Inhibitory Concentration (MIC) of the plant extract (*Artocarpus altilis)* and was then incubated at 37°C for 24 consecutive days to investigate the ability of the selected test isolates to develop resistance. A test-tube containing nutrient broth and the plant extract was also incubated alongside as control. On day 12, 18 & 24, the

antibacterial testing using Agar Diffusion Method was done to check for possible reductions in the zones of inhibition. The control was also plated to assure the purity of the culture (Pai-Wei et al., 2015).

3. STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard deviation (SD) of two replicates.
Statistical significance was determined by student's *t*-test. p-value < 0.05 was considered as significant.

4. RESULTS

4.1. Phytochemical Screening

The result of the qualitative phytochemical screening of the methanol extracts of *Artocarpus altilis* stem bark showed the presence of saponins, tannins, flavonoids, terpenoids, alkaloids, and anthraquinone, as shown in Table 1. Cardiac glycosides and steroids were seen to be absent. The quantitative phytochemical screening indicated that Alkaloids has the highest yield of 35g, as shown in Table 1.

4.2. Antimicrobial Assessment

All the multi-drug bacteria obtained (6) were susceptible to the methanol extract of *Artocarpus altilis* at varying concentrations with a diameter of inhibition (mm) ranging from a mean value of 5.25±0.25 to 21.25±3.25 at 160mg/ml as shown in Table 2. Positive and negative control was used for this experiment where the positive control, 'Ofloxacin,' is a second-generation fluoroquinolone antibiotic that has broad-spectrum activity, and the negative control is Dimethylsulfoxide (DMSO) which is a polar solvent used in dissolving the crude extract.

4.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of the methanol extract of *Artocarpus altilis* on the multi-drug resistant bacteria are presented in Figure 1. The MIC values range from 10mg/ml to 40mg/ml, with *Proteus mirabilis* having the highest MIC (40mg/ml). The MBC of the extract ranges from 10mg/ml to 80mg/ml, with four (4) of the bacterial isolates having MBC of 80mg/ml, as shown in Figure 1.

4.4. Resistance Analysis

The bacteria isolates were exposed to the methanol extract of *Artocarpus altilis* at a concentration lower than the MIC for 24 days, and the resistance pattern of the isolates was tested at day twelve (12), eighteen (18) and twenty-four (24) as shown in Table 3, 4 and 5 respectively.

After 12 days of exposure, the MAR bacteria were seen to be susceptible to the methanol extract of *Artocarpus altilis* at all concentrations (160mg/ml, 80mg/ml, 40mg/ml and 20mg/ml) except for *Pseudomonas aeruginosa* and *Proteus mirabilis* which was not susceptible to the extract at 20mg/ml. On day 18, no clear zone of inhibition was seen for *Proteus mirabilis* at all concentrations. *Pseudomonas aeruginosa* was only susceptible to the extract at 160mg/ml as there was no zone of inhibition for the other concentrations (80mg/ml, 40mg/ml and 20mg/ml), this was after the bacterial isolates have been exposed to a concentration lower than the MIC for eighteen days. In Table 5, all the bacterial isolates after twenty-four days (24) showed no zone of inhibition at 40mg/ml and 20mg/ml except for *Bacillus cereus*, which was still susceptible at the different concentrations although there were reductions in the zone of inhibition compared to the unexposed bacterial isolates.

Table 6 shows the difference in the diameter of inhibition (mm) of the bacterial isolates before exposure and after exposure to a concentration lower than the MIC of the methanol extract of *Artocarpus altilis* and from this table there was a noticeable reduction in the diameters of inhibition (mm) at a concentration of 160mg/ml of all the bacterial isolates except for *Proteus mirabilis* which became completely resistant, i.e., there was no zone of inhibition at this concentration.

Table-1. Qualitative and Quantitative Phytochemical Constituents of *Artocarpus altilis*.

TESTS	Qualitative	Quantitative (g)
Saponins	+	7.7
Tannins	+	ND
Cardiac glycosides	-	ND
Flavonoids	+	5.1
Terpenoid	+	ND
Steroids	-	ND
Alkaloids	++	35
Anthraquinone	+	ND

KEYS: - = Absent + = Present ++ = Strongly present ND = Not Determined.

Table-2. Antimicrobial activity of methanol extract of *Artocarpus altilis* against bacterial isolates.

ISOLATES	DIAMETER OF INHIBITION (mm)				CONTROL	
	160mg/ml	80mg/ml	40mg/ml	20mg/ml	Ofloxacin	DMSO
Staphylococcus epidermidis	21.25±3.25	15.75±0.75	9.25±0.25	8.25±0.25	26.25±1.25	-
Escherichia coli	11.75±1.25	9.75±0.25	7.75±0.25	4.50±0.50	29.50±0.00	-
Staphylococcus saprophyticus	9.25±0.25	8.75±0.25	6.5±0.50	3.5±0.5	24.25±0.25	-
Pseudomonas aeruginosa	9.25±1.75	7.25±0.75	5.00±0.5	4.00±0.00	21.25±2.50	-
Proteus mirabilis	5.25±0.25	3.5±0.00	2.50±0.00	0.00	37.00±1.00	-
Bacillus cereus	11.75±0.75	9.00±0.5	7.00±0.50	5.25±1.25	31.00±1.00	-

Keys: Values are Mean± standard deviation of duplicate observations.

Table-3. Antimicrobial activity of methanol extract of *Artocarpus altilis* against bacterial isolates exposed to a concentration lower than the MIC for twelve days.

ISOLATES	DIAMETER OF INHIBITION (mm)				CONTROL
	160mg/ml	80mg/ml	40mg/ml	20mg/ml	DMSO
Staphylococcus epidermidis	19.00±1.00	17.00±1.00	13.25±2.75	14.00±2.00	-
Escherichia coli	12.75±1.25	9.50±0.00	8.00±0.00	6.00±0.00	-
Staphylococcus saprophyticus	12.50±1.50	9.75±1.25	8.75±0.75	7.00±0.50	-
Pseudomonas aeruginosa	10.50±0.00	8.75±1.25	5.50±0.00	0.00	-
Proteus mirabilis	8.00±0.00	5.50±1.00	4.50±0.50	0.00	-
Bacillus cereus	12.50±2.00	7.75±1.25	6.25±0.75	4.50±0.00	-

Keys: + = Activity; - = No activity ; Values are Mean± standard deviation of duplicate observations.

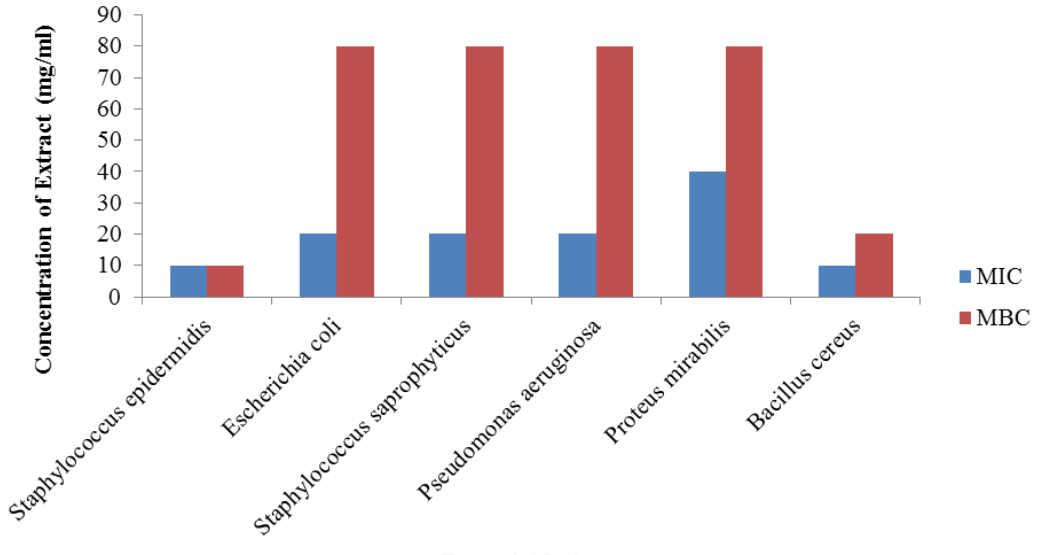

Figure-1. A chart showing the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of methanol extract of *Artocarpus altilis*.

Table-4. Antimicrobial activity of methanol extract of *Artocarpus altilis* against bacterial isolates exposed to a concentration lower than the MIC for eighteen days.

ISOLATES	DIAMETER OF INHIBITION (mm)				CONTROL
	160mg/ml	80mg/ml	40mg/ml	20mg/ml	DMSO
Staphylococcus epidermidis	8.75±0.25	7.15±0.65	3.38±0.48	2.25±0.25	-
Escherichia coli	7.25±0.25	5.25±0.25	2.00±0.50	0.00	-
Staphylococcus saprophyticus	9.00±0.00	8.00±0.50	5.75±0.75	3.50±0.00	-
Pseudomonas aeruginosa	4.00±0.00	0.00	0.00	0.00	-
Proteus mirabilis	0.00	0.00	0.00	0.00	-
Bacillus cereus	8.50±0.50	6.75±0.75	4.88±0.13	3.00±0.00	-

Keys: Values are Mean± standard deviation of duplicate observations.

Table-5. Antimicrobial activity of methanol extract of *Artocarpus altilis* against bacterial isolates exposed to a concentration lower than the MIC for twenty-four days.

ISOLATES	DIAMETER OF INHIBITION (mm)				CONTROL
	160mg/ml	80mg/ml	40mg/ml	20mg/ml	DMSO
Staphylococcus epidermidis	6.50±1.00	4.75±0.75	0.00	0.00	-
Escherichia coli	5.75±0.25	2.75±0.25	0.00	0.00	-
Staphylococcus saprophyticus	9.25±0.25	6.50±0.50	0.00	0.00	-
Pseudomonas aeruginosa	6.25±0.25	0.00	0.00	0.00	-
Proteus mirabilis	0.00	0.00	0.00	0.00	-
Bacillus cereus	7.75±0.25	6.50±1.00	5.00±0.50	3.62±1.86	-

Keys: Values are Mean± standard deviation of duplicate observations.

Table-6. Difference in Diameter of Inhibition (mm) at 160mg/ml of Isolates before exposure to sub-lethal concentration and after exposure for 24 days to *Artocarpus altilis*.

Isolates	Diameter Of Inhibition (Mm)	
	Before Exposure	After Exposure
Staphylococcus epidermidis	21.25±3.25	6.50±1.00
Escherichia coli	11.75±1.25	5.75±0.25
Staphylococcus saprophyticus	9.25±0.25	9.25±0.25
Pseudomonas aeruginosa	9.25±1.75	6.25±0.25
Proteus mirabilis	5.25±0.25	0
Bacillus cereus	11.75±0.75	7.75±0.25

Keys: Values are Mean± standard deviation of duplicate observations.

5. DISCUSSION

Plants are rich in an extensive variety of secondary metabolites such as tannins, alkaloids, terpenoids, and flavonoids which possess antimicrobial properties and may serve as an alternative, effective, cheap and safe antimicrobial for the treatment of microbial infections (Pandey & Kumar, 2013; Shaikh & Patil, 2020).

Six (6) multi-drug resistant organisms were utilized for this study; these organisms were identified to be *Staphylococcus epidermis*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. The presence of phytochemicals such as saponins, tannins, flavonoids, terpenoids, alkaloids, and anthraquinone in the methanol extract of the stem bark of *Artocarpus altilis* confirmed its effectiveness as an antibacterial agent, and this is in agreement with the work of Pradhan et al. (2013) who reported that the fruit extracts of *Artocarpus altilis* have the immense potentiality for antibacterial activities as a result of the presence of phytochemicals such as steroids, phenols, and flavonoids which play a significant role in preventing the growth of human pathogens and act against them by developing an effective defense mechanism.

The result of the antimicrobial sensitivity of the methanol extract of the stem bark of *Artocarpus altilis* in this study showed different zones of inhibition to the test bacterial isolates. All the test isolates which were already multi antibiotics resistance were susceptible to the extract at different concentrations (160mg/ml, 80mg/ml, 40mg/ml and 20mg/ml). This is in agreement with the work of Felipe et al. (2018) who worked on antibacterial activity of Lamiaceae plant extracts in clinical isolates of multi-drug resistant *Acinetobacter baumanii*, *Klebsiella*

pneumonia, *Escherichia coli* and *Pseudomonas aeruginosa* which were resistant to three (3) classes of antibiotics and reported that the ethanol extract of *Plectranthus barbatus* showed activity against strains of all the test bacterial used.

From the result, the highest diameter zone of inhibition for methanol stem bark extract of *Artocarpus altilis* is 21.25±3.25 in *Staphylococcus epidermidis* while the least inhibition of 5.25±0.25 against *Proteus mirabilis* at 160mg/ml.

The Minimum Inhibitory Concentrations range from 10mg/ml to 40mg/ml while the Minimum Bactericidal Concentration ranges from 10mg/ml to 80mg/ml. This suggests that the extract has both bactericidal and bacteriostatic activity.

The result of the antimicrobial activity of methanol stem bark of *Artocarpus altilis* after exposure for twenty-four (24) days shows that bacteria can develop resistance to plant antimicrobial. After twelve (12) days of exposure, there was a reduction in the zone of inhibition at different concentrations. After exposure for eighteen (18) days, *Proteus mirabilis* became utterly resistant to the extract as there were no visible zones of inhibition. After 24 days of exposure, *Bacillus cereus* was still susceptible at the lowest concentration (20mg/ml), *Pseudomonas aeruginosa* was only susceptible at the highest concentration (160mg/ml) while the remaining bacterial test isolates became resistant at 40mg/ml and 20mg/ml. This result is in agreement with the work of Ruiz (2017). They reported that *Staphylococcus aureus* was able to develop resistance to antimicrobial plant extracts, which have previously been shown to have antimicrobial activity and have a strong history of traditional use against bacterial infection. The results obtained from this study showed the possibility of bacteria developing resistance to plant antimicrobials after long exposure at a concentration lower the Minimum Inhibitory Concentration which is in disagreement with the work of Pai-Wei et al. (2015) who reported that exposure of bacteria to a concentration lower than the MIC of antimicrobial plant extract does not induce resistance. This result is also in accordance with the work of Vadhana et al. (2015) who reported that there is some insensitivity or resistance in microbes towards some common herbal antimicrobial compounds, although their mechanism of resistance is not precise.

6. CONCLUSION

This study demonstrated that exposure of bacteria to antimicrobial plant extract at a sub-lethal concentration could induce the development of resistance which was observed by a reduction in Minimum Inhibitory Concentration (MIC) and Diameter of zones of inhibition (mm) of the organisms exposed to sub-lethal concentration compared to the same organisms before exposure. Hence, there is need for proper use of antimicrobials from plant extracts to avoid the development of resistance to plant antimicrobials and also to avoid similar problems currently faced with conventional antibiotics.

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