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ANTIBACTERIAL POTENTIAL OF PARTIALLY PURIFIED FRACTIONS OF LEAF EXTRACT OF *SPONDIAS MOMBIN*

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Keywords:

Antibacterial activity, Partially purified fractions, *Spondias mombin*, Pathogenic bacteria, Killing rate

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ABSTRACT: This study was conducted to investigate the antibacterial activity and rate of kill of the various partially purified fractions of *Spondias mom*bin's leaf extract on selected pathogenic bacteria. Fresh leaves of *Spondias mombin* were air-dried and then ground into fine powder. The powdered leaves of *Spondias mombin* were extracted using ethanol-water in the ratio 1:1 (v/v). The crude extract was fractionated by partial purification using various solvents such as n-hexane, chloroform, ethyl-acetate, and n-butanol. The fractions were concentrated *in vacuo* and lyophilized. Afterward, the different fractions were tested for antibacterial activities against selected pathogenic bacteria using the agar well diffusion method. The minimum inhibitory and minimum bactericidal concentrations were determined using the standard microbiological method. The rate of kill of the test isolates by the active fractions (n-butanol, ethyl acetate, and n-hexane fractions) was also determined. The zones of inhibition shown by the active fractions at 15 mg/mL on the isolates ranged between 11.7 mm and 15.7 mm while the minimum inhibitory and minimum bactericidal concentrations of the active fractions on the bacterial isolates were 15.00 mg/mL. The killing/death rate of the active fractions was observed to increase with increased concentration of the active fractions and increased contact time. This study established that the fractions of *Spondias mombin* leaf extract investigated had considerable antibacterial activity and were bactericidal in action with a broad spectrum of activity against both Gram-negative and Gram-positive bacteria used in this study.

INTRODUCTION: *Spondias mombin* Linn. (Anacardiaceae) is a fructiferous tree having a habitat in Nigeria, Brazil, and several other tropical forests and coastal area in the world. It can reach a height of 15-22m. It is very rich in vitamins B_1 and C. The fruit mostly exists as an oval seed.

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This plant is common around us in South West of Nigeria (Yoruba) and is commonly used in folk medicine. Concerning the popular use of this plant, the leaves were reported in the literature to possess antimicrobial and antiradical activities. It has also been reported that the aqueous, methanolic and ethanolic extracts of the leaves of *Spondias mombin* have antihelminthic properties, pronounced antibacterial properties against *Bacillus cereus*, *Streptococcus pyogenes*, and *Mycobacterium fortuitum* [1-3]. The ethnopharmacology associated with the chemical study has become an important tool in bioprospecting.

Studies have associated information on the traditional use of medicinal plants with phyto-chemical and pharmacological studies, searching for new drugs and herbal medicines [4].

Plants perform as a renewable natural resource of diverse bioactive compounds. Plant extracts consist of various compounds characteristic to the plant from which they were extracted. Aromatic secondary metabolites synthesized by plants are phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, and coumarins [5].

In traditional methods of drug discovery, plant materials are tested for pharmaceutical purposes. If any evidence of activity is observed, the extract is fractioned, and the active compound is isolated and identified. Each step of decomposition and isolation is usually guided by biological tests, which is referred to as bioassay-guided fractionation [6]. The World Health Organization reports that at least 75-95% of the world populations of developing countries chiefly rely on traditional medicine, and major parts of traditional therapies involve the use of plant extract and or their active constituents [7].

MATERIALS AND METHODS:

Collection of Clinical and Standard Strains: The microorganisms namely; *Escherichia coli, Shigella sonnei, Salmonella typhimurium, Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus* and *Bacillus stearothermophilus* were obtained from the Microbiology Laboratory of Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. These organisms include two standard strains of the National Collection of Industrial Bacteria (NCIB); *Escherichia coli* (NCIB 86) and *Staphylococcus aureus* (NCIB 8588). All other thirteen isolates were locally isolated organisms (LIO). The isolated identities were reconfirmed by cultural and conventional biochemical tests with reference to Bergey's Manual of Determinative Bacteriology. The cultures were maintained on a nutrient agar slant and stored at 4 °C until further use.

Plant Sample Collection and Authentication: The leaves of *Spondias mombin* used in this study were collected in November 2014 along Road 7, Ile-Ife, Osun State, Nigeria. The sample was identified at the Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, with voucher number: IFE –17456. The voucher specimen was deposited for reference purposes.

Drying and Extraction of the Plant Sample: The leaves of *Spondias mombin* were air-dried under the shade at room temperature. This was ground into a fine powder using an electric blender. The powdered leaves (1525g) were soaked in the mixture of ethanol and sterile distilled water in ratio 1:1 (v/v) for 72 hours with regular agitation and then filtered using number 1 Whatman filter paper. The filtrate was concentrated *in vacuo* using rotary evaporator and lyophilized. The yield obtained was 97.18g, and the extract was amber in colour.

Solvent Partitioning of the Leaf Extract of *Spondias mombin*: Exactly 50g of crude extract of *Spondias mombin* was mixed in 300 ml of sterile distilled water and poured into a separatory funnel, and extracted with n-hexane. The resulting n-hexane fraction was concentrated to dryness *in vacuo,* and the residue (14.50 g) was kept in a freezer in an air-tight container. The resultant aqueous phase was re-concentrated *in-vacuo* to remove traces of n-hexane. The residue was further extracted with chloroform. The chloroform fraction obtained was also concentrated *in vacuo* to dryness, and 1.38 g powder collected was kept in the freezer for further use. The ethyl acetate (17.12 g) and butanol (13.01 g) fractions were also obtained using a similar procedure. The remaining aqueous fraction was freeze-dried to yield 2.85 g powder which was kept in the freezer for further use.

Antibiotic Susceptibility Testing on the Test Isolates: The susceptibility of the isolates to some conventional antibiotics was determined using the disc diffusion technique. Bacterial isolates were first grown in nutrient broth for 18 hours before use. About 0.2 ml of the standardized test isolates (10^6cfu/ml of 0.5 McFarland standard) were then sub-cultured onto Mueller-Hinton agar (Oxoid, UK). The medium was allowed to set. The agar plate was swabbed with a previously standardized inoculum using a sterile swab stick. Sterile forcep was used to place the multiple antibiotic discs (Gram-positive and Gram-negative discs) on separate seeded plates. The plates were then inverted and incubated at 37 °C for 24 hours. The

diameter of zones of inhibition was measured with a transparent ruler to the nearest millimeter and compared to CLSI [8] interpretative chart of zones of inhibition of antibiotics.

Sensitivity Testing of the Fractions on Bacterial Isolates: The sensitivity testing of the various fractions (n-butanol, ethyl acetate, chloroform n-hexane, and aqueous fractions) was determined using the agar-well diffusion method as described by Hugo and Russel [9]. The bacterial isolates were first grown in nutrient broth for 18 hours before use. Exactly 0.2 ml of the standardized test isolates (10^6 cfu/ml of 0.5 McFarland standards) were then sub-cultured onto Mueller-Hinton agar (Oxoid, UK). The medium was allowed to set, and wells were then bored into the agar medium using a sterile 6 mm cork borer. The wells were then filled up with the prepared solution of the extract (15 mg/mL) and care was taken not to allow solution to spill on the surface of the medium. The plates were allowed to stand on the laboratory bench for about 1-2 h to allow proper inflow of the solution into the medium before incubating at 37 °C for 24 h. The plates were observed for the zones of inhibition. The diameter of zones of inhibition was measured by a transparent ruler to the nearest millimeter.

Determination of Minimum Inhibitory Concentrations (MIC) of the Fractions against Bacterial Isolates: The MIC of the fractions was determined using the method described by Akinpelu *et al*. [10] Two-fold serial dilution of the extract was prepared using 15 mg/mL of the crude extract. Exactly 2 ml of different concentrations of the solution was added to 18 ml of pre-sterilized molten nutrient agar to give final concentrations of 0.4688 to 15.0 mg/mL. The medium was then poured into sterile Petri dishes and allowed to set. The surfaces of the media were allowed to dry before streaking with 18 hours old standardized bacterial cultures grown in sterile nutrient broth. The plates were incubated at 37 °C for up to 72 hours, after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevented the growth of the bacteria.

Determination of Minimum Bactericidal Concentrations (MBC) of Fractions against Bacterial Isolates: The MBC of the fractions was determined by the method of Spence and Spencer [11]. Samples were taken from plates with no visible growth in the MIC assay and sub-cultured onto freshly prepared nutrient agar plates and later incubated at 37 °C for 48 hours. The lowest concentration of the extract that did not show any growth on a new set of plates was taken as the minimum bactericidal concentration of the extract.

Determination of the Rate of Kill of the Active Fractions on the Test Isolates: The rate of the kill was performed using the method described by [12]. An experiment was determined using each of the fractions on the viability of *Escherichia coli* representing Gram-negative and *Staphylococcus aureus* representing Gram-positive organisms. A viable count of the test organisms was initially determined. Exactly 0.5 ml volume of known cell density (10^6 cfu/ml by viable counts) from each organism suspension was added to 4.5 ml of different concentrations of the fractions. The suspension was thoroughly mixed and held at room temperature (28-30 °C), and the killing rate was determined over a period of 2 hours. Exactly 0.5 ml of each suspension was withdrawn at the appropriate time intervals and transferred to 4.5 ml nutrient broth (Oxoid, UK) recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compounds carry-over from the test suspensions. The suspension was shaken properly, then serially diluted up to 10^{-5} in sterile physiological saline. Exactly 0.5 ml of the final dilution of the test organism was transfer into pre-sterile nutrient agar (Oxoid Ltd) at 45 °C and plated out. The plates were allowed to set and incubated upside down at 37 °C for 72 hours. The control experiment was set up without the inclusion of antimicrobial agent. Viable counts were made in triplicates for each sample. Reduction in the viable counts indicated killing by the antimicrobial agent.

Statistical Analysis: All data were expressed as mean ± standard deviation (SD) of three replicates and were statistically analyzed using analysis of variance (ANOVA). The difference was considered significant at $P<0.05$.

RESULTS:

Antibacterial Activities Exhibited by the Active Fractions: The result of antibacterial susceptibility testing of the various fractions against the test isolates revealed a zone of inhibition ranging from

11.7 mm to 15.7 mm in diameter at 15 mg/mL. The butanol fraction was most active against *Escherichia coli*, and least active against *Shigella sonnei* 001, having zones of inhibition of 15.0 mm and 11.0 mm respectively. Ethyl acetate had a zone of inhibition of 15.7 mm against *Bacillus stearothermophilus,* and 12.0 mm against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* 002, *Pseudomonas aeruginosa* and *Shigella Sonnei* 002. The n-hexane fraction, on the other hand, exhibited a zone of inhibition of 15.0 mm and 12.0 mm against *Escherichia coli* and *Shigella sonnei* 002, respectively **Table 1**. The isolates were found to be resistant to multiple antibiotic discs, as indicated in **Table 2** and **Table 3**.

TABLE 1: ANTIBACTERIAL SENSITIVITY PROFILE EXHIBITED BY THE ACTIVE FRACTIONS

	Zone of inhibition (mm) **		
Bacterial Isolates	**N-butanol fraction 15mg/mL**	**Ethyl acetate fraction 15 mg/mL**	**N-hexane fraction 15mg/mL**
E. coli	15.0 ± 1.0	13.0 ± 0.0	13.3 ± 1.5
E. coli (NCIB 86)	12.7 ± 0.6	12.0 ± 0.0	15.0 ± 0.0
K. pneumonia	13.0 ± 0.0	12.0 ± 1.0	12.0 ± 1.0
P. vulgaris 001	14.0 ± 1.0	13.7 ± 0.6	14.0 ± 1.0
P. vulgaris 002	12.7 ± 0.6	12.0 ± 1.0	13.7 ± 0.6
P. mirabilis	14.3 ± 0.6	12.3 ± 1.5	12.7 ± 0.6
P. aeruginosa	14.0 ± 0.0	12.0 ± 0.0	13.0 ± 0.0
S. typhi (001)	15.0 ± 1.0	14.7 ± 0.6	14.0 ± 0.0
S. typhi (002)	12.7 ± 0.6	13.3 ± 0.6	13.0 ± 1.0
S. sonnei (001)	11.0 ± 0.0	13.0 ± 0.0	13.0 ± 0.0
S. sonnei (002)	11.7 ± 0.6	12.0 ± 0.0	12.0 ± 0.0
S. aureus	12.7 ± 0.6	13.3 ± 0.6	14.0 ± 1.0
S. aureus (NCIB 8588)	12.0 ± 1.0	13.0 ± 0.0	14.0 ± 1.0
B. cereus	12.0 ± 0.0	13.0 ± 1.0	14.0 ± 2.0
B. stearotherm.	12.7 ± 0.6	15.7 ± 0.6	14.7 ± 1.5

Key: **: mean of three replicates, *E. coli = Escherichia coli, K. pneumoniae = Klebsiella pneumoniae, P. vulgaris = Proteus vulgaris, P. mirabilis = Proteus mirabilis, P. aeruginosa = Pseudomonas aeruginosa, S. typhi = Salmonella typhimurium, S. sonnei = Shigella sonnei, S. aureus = Staphylococcus aureus, B. cereus = Bacillus cereus, B. stearotherm. = B. stearothermophilus*, NCIB: National Collection of Industrial Bacteria.

The Minimum Inhibitory and Bactericidal Concentrations: The MIC and MBC exhibited by each of the three active fractions were 15 mg/mL, but the MBC of some of the isolates were determined at the same concentration (15 mg/mL) **Table 4**.

TABLE 2: MINIMUM INHIBITORY AND MINIMUM BACTERICIDAL CONCENTRATIONS OF THE ACTIVE FRACTIONS

	Zone of inhibition (mm) **					
	N-butanol fraction		**Ethyl acetate fraction**		**N-Hexane fraction**	
Bacterial Isolates	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**
E. coli	15.00	15.00	15.00	ND	15.00	ND
E. coli (NCIB 86)	15.00	15.00	15.00	ND	15.00	ND
K. pneumoniae	15.00	15.00	15.00	15.00	15.00	15.00
P. vulgaris 001	15.00	15.00	15.00	ND	15.00	15.00
P. vulgaris 002	15.00	ND	15.00	ND	15.00	15.00
P. mirabilis	15.00	15.00	15.00	15.00	15.00	15.00
Ps. aeruginosa	15.00	ND	15.00	15.00	15.00	15.00
S. typhi 001	15.00	ND	15.00	ND	15.00	ND
S. typhi 002	15.00	ND	15.00	15.00	15.00	15.00
S. sonnei 001	15.00	ND	15.00	15.00	15.00	15.00
S. sonnei 002	15.00	ND	15.00	ND	15.00	15.00
S. aureus	15.00	ND	15.00	ND	15.00	15.00
S. aureus (NCIB 8588)	15.00	ND	15.00	ND	15.00	ND
B. cereus	15.00	15.00	15.00	ND	15.00	15.00
B. *stearotherm.*	15.00	ND	15.00	ND	15.00	15.00

Key: **: *E. coli = Escherichia coli, K. pneumoniae = Klebsiella pneumoniae, P. vulgaris = Proteus vulgaris, P. mirabilis = Proteus mirabilis, P. aeruginosa = Pseudomonas aeruginosa, S. typhi = Salmonella typhimurium, S. sonnei = Shigella sonnei, S. aureus = Staphylococcus aureus, B. cereus = Bacillus cereus, B. stearotherm. = B. stearothermophilus*, NCIB: National Collection of Industrial Bacteria, ND = Not determined

The Kill/Death Rate Assay: The kill/death rate assay of the active fractions was carried out against bacterial test isolates as shown in **Fig. 1-6**. The rate of kill of Escherichia coli by n-butanol, ethyl acetate, and n-hexane fractions at the MIC 1 after 15 minutes of the contact time interval with the active fractions were 6.5%, 4.3%, and 5.2%, respectively, while it was 14.3%, 13.0%, and 9.5% respectively at 30 minutes. The killing rate continues to increase progressively at the same concentration as the contact time increased. The killing rate was 68.8%, 65.4%, and 64.1% for n-butanol, ethyl acetate, and n-hexane fractions, respectively, after 120 minutes. When the concentration was doubled (*i.e.* 2 x MIC), 15.6%, 30.3%, 44.6%, 66.2%, 93.9%, of the bacteria were killed by n-butanol fraction, 6.9%, 16.0%, 29.4%, 58.4%, 79.2% of the bacteria were killed by ethyl acetate while 10%, 19%, 35.1%, 65.8%, 81.8%, of the bacterial cells were killed at 15, 30, 60, 90 and 120 minutes, respectively. When the concentrations of the fractions were tripled (*i.e.*, 3 x MIC), none of the bacteria (*Escherichia coli*) survived after 60 minutes of the contact time with n-butanol fraction, while no growth of the organisms occurred at 90 minutes for both ethyl acetate and n-hexane fractions **Fig. 1-3**.

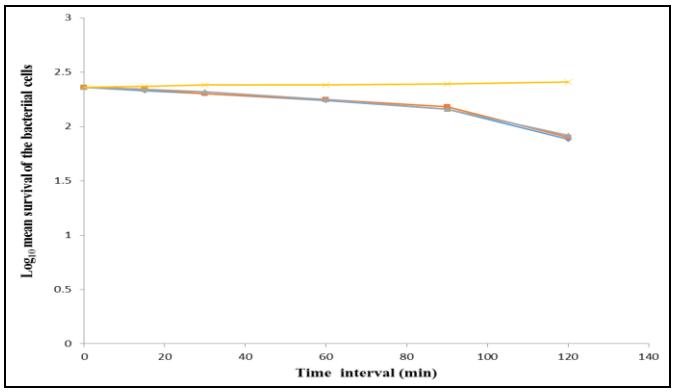

FIG. 1: THE EXTENT AND RATE OF KILLING OF *ESCHERICHIA COLI* BY N-BUTANOL, ETHYL ACETATE AND N-HEXANE FRACTIONS AT MIC 1 Key: = N- butanol, = Ethyl acetate, = N-hexane, = Control

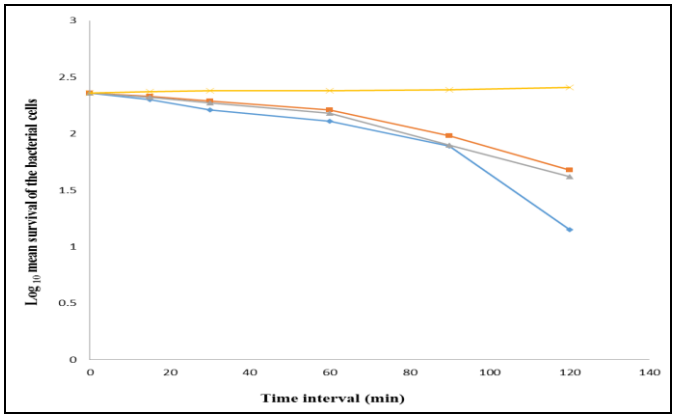

FIG. 2: THE RATE AND EXTENT OF KILLING OF *ESCHERICHIA COLI* BY N-BUTANOL, ETHYL ACETATE AND N-HEXANE FRACTIONS AT DOUBLE MIC (2 X MIC). Key: = N- butanol, = Ethyl acetate, = N-hexane, = Control

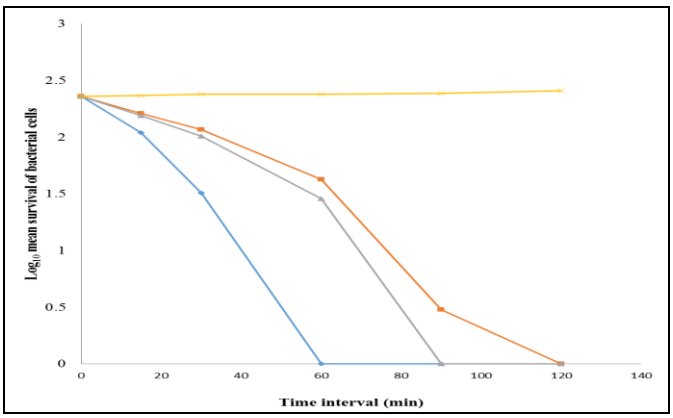

FIG. 3: THE RATE AND EXTENT OF KILLING OF *ESCHERICHIA COLI* BY N-BUTANOL, ETHYL ACETATE AND N-HEXANE FRACTIONS AT 3 X MIC. Key: = N-butanol, = Ethyl acetate, = N-hexane, = Control

At MIC 1, the killing rate of *Staphylococcus aureus* by butanol fraction was 9.5%, 21.6%, 32.6%, 43.9%, and 64.0% at 15, 30, 60, 90 and 120 minutes contact time respectively. At the same concentrations, the killing rate exhibited by ethyl acetate fraction was 11.7%, 22.3%, 37.1%, 51.9%, 69.7% while 12.1%, 22.3%, 35.6%, 53.8% and 77.3% killing was achieved by the n-hexane fractions at 15, 30, 60, 90 and 120 minutes respectively. The killing rate of the fractions was observed to be increasing with increased concentrations of the fractions and time interval. Meanwhile, 100% killing was exhibited on *Staphylococcus aureus* by n-butanol, ethyl acetate and n-hexane fractions at 120, 90 and 60 minutes contact time, respectively **Fig. 4-6**.

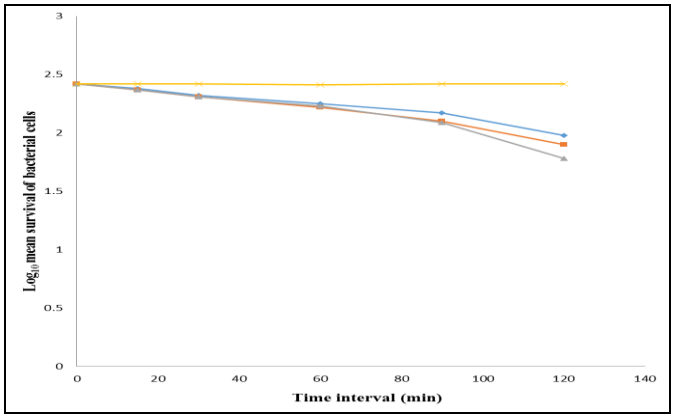

FIG. 4: THE EXTENT AND THE RATE OF KILLING OF *STAPHYLOCOCCUS AUREUS* BY N-BUTANOL, ETHYL ACETATE AND N- HEXANE AT MIC 1. Key: = N- butanol, = Ethyl acetate, = N-hexane, = Control

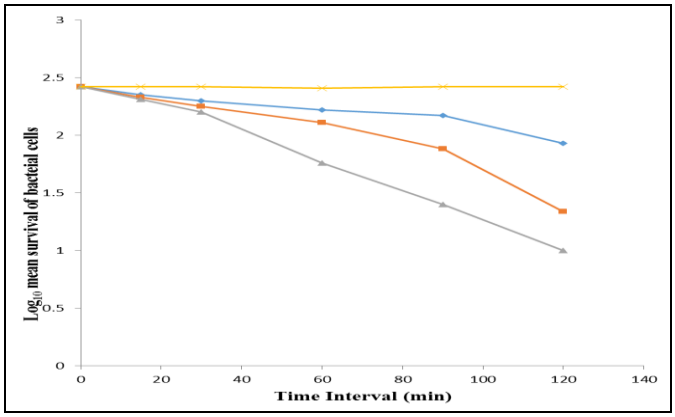

FIG. 5: THE EXTENT AND RATE OF KILLING OF *STAPHYLOCOCCUS AUREUS* BY N-BUTANOL, ETHYL ACETATE AND N-HEXANE AT DOUBLE MIC (2 X MIC). Key: = N- butanol, = Ethyl acetate, = N-hexane, = Control

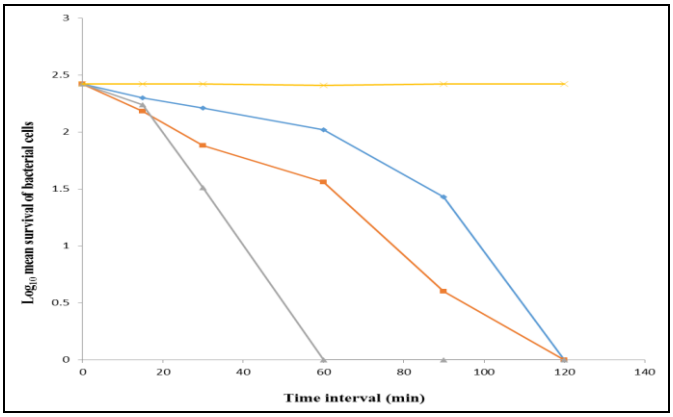

FIG. 6: THE EXTENT AND RATE OF KILLING OF *STAPHYLOCOCCUS AUREUS* BY N-BUTANOL, ETHYL ACETATE AND N-HEXANE AT TRIPLE MIC (3 X MIC). Key: = N- butanol, = Ethyl acetate, = N-hexane, = Control

DISCUSSION: In this study, at a concentration of 15.00 mg/mL, all the bacterial isolates were susceptible to the active fractions (n-butanol, ethyl acetate, and n-hexane fractions). The zones of inhibition shown by the fractions at 15 mg/mL on the isolates ranged between 11.7 mm and 15.7 mm **Table 1**. The two other fractions (chloroform and aqueous fractions) showed no activity. The three active fractions were effective against all the Gram-positive and Gram-negative organisms used in this study even at a low concentration (15 mg/mL), which shows that the extract from *Spondias mombin* is very potent and can serve as a potential broad-spectrum antibacterial drug in the treatment of bacterial infections caused by the bacterial pathogens.

The MIC and MBC exhibited by each of the three active fractions was 15 mg/mL, while the MBC of some of the isolates were determined at the same concentration (15 mg/mL). According to Holezt *et al.*, [13] extracts exhibiting lower MIC (MIC less than 100 mg/mL) has a good activity; thus, the lower MIC values **Table 2** exhibited by the n-butanol, ethyl acetate, and n-hexane fractions of *Spondias mombin* extract against the test isolates suggests it to be a veritable source of bioactive compound. Nostro *et al.*, [14] reported that the ability of an antibacterial agent to inhibit and kill bacteria is a measure of its effectiveness. This further supports the fact that the extract from this plant may be useful in the production of drugs for the treatment of various bacterial infections associated with the test isolates.

The active fractions used for elimination of the bacterial cells achieved 100% at a low concentration within a short time. The ability of Spondias *mombin* leaf extract to kill the test isolates within the shortest period of time indicates a better bactericidal effect. This finding supported the investigations conducted by Pankey and Sabath [15]. The killing of the test bacterial isolates could be attributed to damage caused to the cytoplasmic

membrane of these organisms. Damage to the cytoplasmic membrane will lead to the leakage of protoplasmic inclusion.

CONCLUSION: In conclusion, the study confirmed that n-butanol, ethyl acetate, and n-hexane fractions of the leaf extract of *Spondias mombin* investigated possessed antibacterial property with a broad spectrum of activity against test Gram-negative and Gram-positive bacterial isolates. The active fractions were found to be bactericidal in action. The rate of kill of the bacterial isolates by the extract increased with increased concentration and contact time. Therefore, the leaves could be formulated to serve as an antibacterial agent in the treatment of various bacterial infections caused by the test isolates.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest

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