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Pre/Post-Plasmid Profile Analysis, Killing- Kinetics and Secondary Metabolites Screening Of *Adenopus breviflorus* (*Benth*) Fruit Extract Against Multiple Drug Resistant Isolates Using *Staphylococcus aureus* (*MDRSA*) as a Case Study

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Abstract

This research work was designed to evaluate the pre-plasmid and post-plasmid, killing kinetics assay and secondary metabolites analysis of *Adenopus breviflorus (Benth) (Lagenaria breviflora roberty)* against multiple drug resistant isolates using Staphylococcus aureus as a case study. Multiple drug resistant isolates were subjected to antibacterial susceptibility testing before plasmid curing and Staphylococcus aureus was used as a case study after plasmid curing using Sodium Dodecyl Sulfate intercalating dye following previously determined standard procedures. Antibacterial susceptibility testing of the bacterial isolates was carried out with ethanol extract of *Adenopus breviflora (Benth)* using agar diffusion techniques as the standard procedures. The agar diffusion was used to test the antibacterial potentials of the extract at different concentrations of 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml. The extracts were tested against three (3) Gram positive and seven (7) Gram negative bacteria. Minimum Inhibitory Concentration (MICs) and Minimum bactericidal Concentration (MBCs) were determined and MIC and MBC values of 12.5 to 25 mcg/ml and 23 to 50mcg/ml respectively was recorded and the time-kill kinetics profile of multiple drug resistant Staphylococcus aureus treated with different concentration of ethanolic extract of *Adenopus breviflorus (Benth)* in different time ranges showed reduction in number of viable cells. The qualitative and quantitative secondary metabolites screening of *Adenopus breviflorus (Benth)* whole fruit ethanol extract revealed the presence of alkaloids, flavonoids, cardiac glycosides, tannins, steroids, saponins, tannins, anthraquin, pyrrolidizine alkaloid and reducing sugars as well as the value of each secondary metabolites in quantity while the presence of the volatile oil was not determined. These compounds are responsible for this broad antibacterial activity.

Keywords: Pre-Plasmid; Post-Plasmid Profile Analysis; Killing-Kinetics; Secondary Metabolites Screening; *Adenopus Breviflorus (Benth);* Lagenaria Breviflora Roberty; Multiple Drug Resistant Isolates; *Staphylococcus aureus*(*MDRSA*)

Introduction

Plasmid curing is the process by which plasmids are removed from bacterial populations. This is an attractive strategy to combat AMR as it has the potential to remove ARGs from a population while leaving the bacterial community intact. Alternatively, a plasmid curing agent could be given to a patient prior to surgery, to reduce the likelihood of a resistant hospital acquired infection. Plasmid curing agents could also be taken by international travelers to reduce the global spread of AMR. In fact, there are very few curing mechanisms that have been tested in vivo, even in experimental models. Therefore, research in this area is urgently needed. Recently, it was shown that 24% of non-antibacterial drugs impact growth of members of the human micro biome [1]. Studies such as this would be important for determining any impact of anti-plasmid compounds on the micro biome. Anti-plasmid strategies alone may solve' AMR and they could play an important role in reducing global resistance levels. Removing drug-resistance plasmids is a strategy for all sectors to reduce the overall burden of AMR [2]. Many compounds have shown some plasmid curing activity. These include detergents, sodium dodecyl sulphate (SDS), biocides, DNA intercalating agents, antibiotics (e.g. aminocoumarins, quinolones, and rifampicin), ascorbic acid, psychotropic drugs (e.g. chlorpromazine) and plant-derived compounds. The effectiveness of these compounds varies greatly and depends on bacterial strain, plasmid and growth conditions. Methods to effectively and safely cure plasmids have the potential to diminish the severity of the impact of drug-resistant infections which will be dealt with during this research work.

Adenopus breviflorus (Benth) is a tree plant, commonly known as "lagenaria breviflora Roberty", belongs to the family of Cucurbitaceae (Gourd family) [1]. It is commonly called Wild

colocynth in English language, in Nigeria, different tribal groups have their indigenous names, in Ibo language: Ogbenwa and in Yoruba language: Tagiri. [2] It is a perennial, seasonal creeping tendril climber. It would usually lie on the ground for want of something to climb and climbs over shrubs and herbs by means of axillary tendrils. Ascending to the forest canopy the leaves are simple, alternate and palmately veined, scabrid and sand papery [3]. The fruit (bulb) is a pepo and appear green with cream-colored narrow blotches measuring 1-5 cm in length and its pulp is bitter [4]. The seeds number up to four hundred in an average – size fruit. The flowers are actinomorphic and nearly always unisexual [5].The stem when crushed has an unpleasant smell and a decoction from it is said to be used in Africa for headache and as a vermifuge [6]. The family is a diverse family of plants in the temperate zones but also thrives in hot arid regions of the world It occurs from Senegal to Western Cameroons and generally widespread in tropical Africa [7] (figure 1)

Figure 1: Source (8)

Indigenously, *Adenopus breviflorus (Benth)* has a long medicinal history values for treating various conditions in Nigeria. The fruits are major important items of trade in Western region of Nigeria and they are of immense values in curative and preventive control measures against conditions such as measles, chickenpox, intestinal worms, enteritis (diarrhea), *Diabetes mellitus,* Newcastle diseases, leather preservative, as wound antiseptics (umbilical incision wound) and as depilatory agent [8, 9].

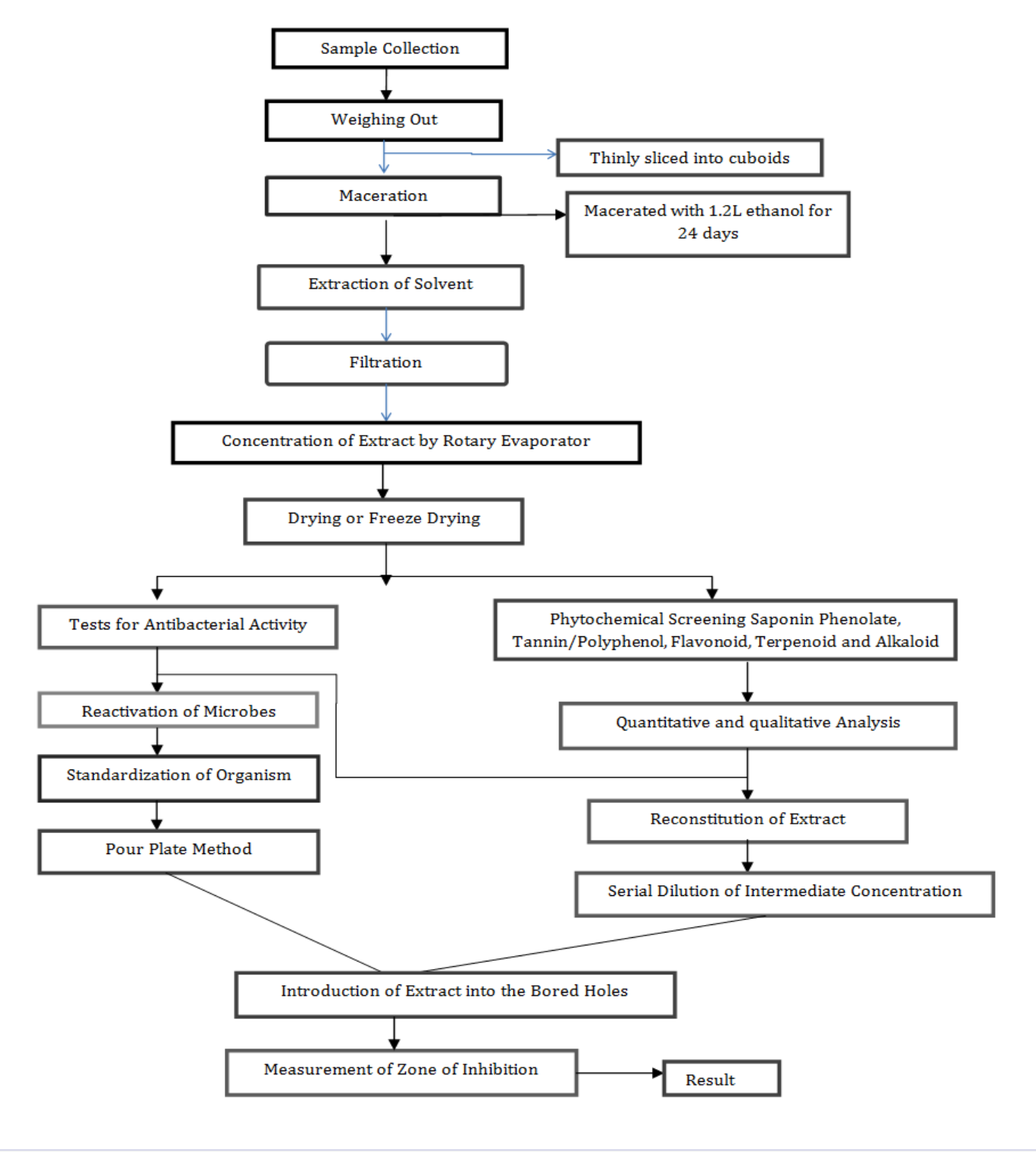
It has been reported that the methanol extract of its whole fruit has anti-implantation activity [10] and abortifacient activity [11]. The ethanol extract of its whole fruit has been reported to have a broad spectrum antibacterial activity [12] as well as anti-oxidant and anti-ulcerogenic effects [13]. The ethanol extract of its whole fruit has been reported to cause increase in RBC, TWBC, PCV values as well as caused electrolytes imbalances [14] and spermatotoxic effect in rats [15]. *Adenopus breviflorus (Benth)* fruits antimicrobial diverse effects, including the earliest antibacterial activity [16, 17] and antiviral activity [18]. While Soxhlet ethanolic extracts exhibited stronger activities against pathogenic bacteria isolates (*Salmonella typhi, Salmonella paratyphi, Pseudomonas fluorescensi*), Steeped extracts showed higher resistance to Shigella dysenteriae, *Shigella flexneri,* and *Pseudomonas fluorescens* [19]. Extracts from *Adenopus breviflorus (Benth)* have been identified as potent anti-inflammatory agents [20, 21, 22, 23].

Fruits and seeds of *Adenopus breviflorus (Benth)* were reported to possess miracicidal and cercaricidal substances effective for controlling transmission of Schistosomiasis [24]. In a related development. *Adenopus breviflorus* (*Benth*) fruit applied as phytobiotics improved the growth performance of broiler and exhibited excellent control of *Eimeria oocyst and Ascaris galli* [25]. The extract demonstrated antioxidant activity by its ability to: quench free radicals generated by nitric oxide and superoxide anion with a concomitant scavenging potential against DPPH-induced radical formation [26], and enhance the recovery from oxidative stress [27].

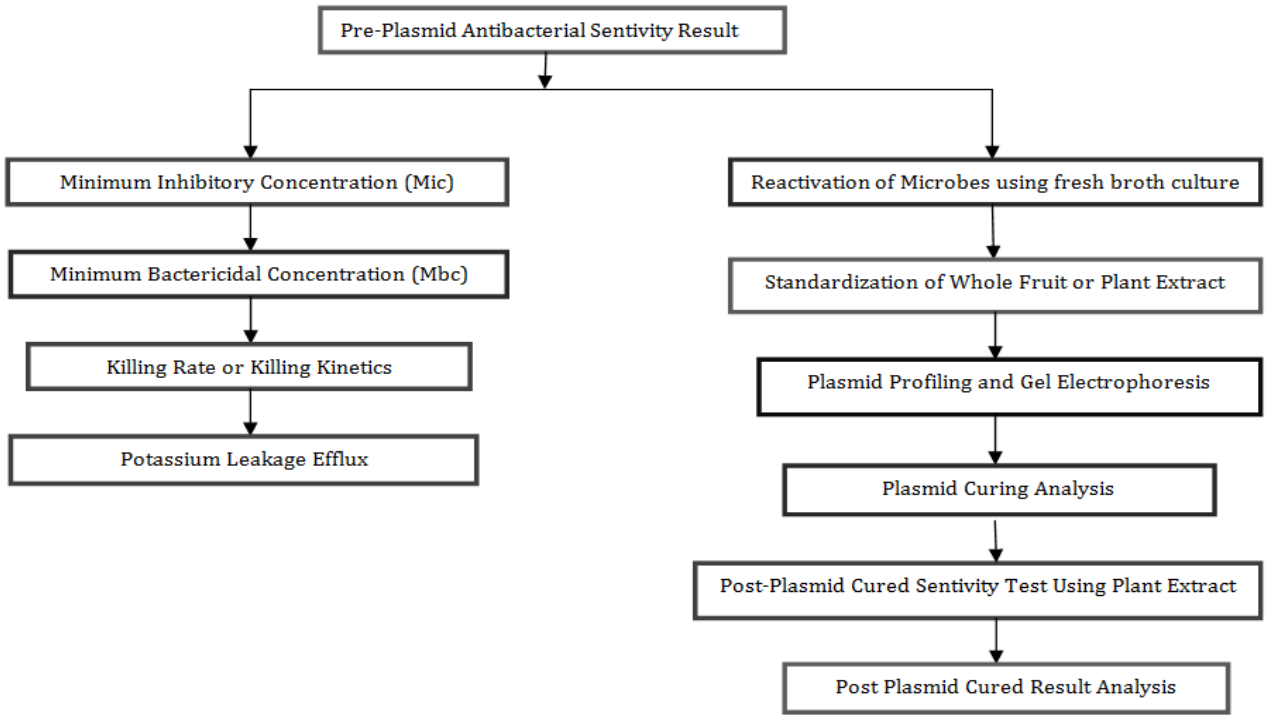
The Framework of this Research

Flow Chart 1: Procedures for antibacterial susceptibility testing of *Adenopus breviflorus (Benth)* against multiple resistant bacteria isolate

Flowchart 2: Procedure for plasmid profiling analysis of multiple resistant *Staphylococcus aureus* using *Adenopus breviflorus (Benth)*



Flow Chart 1: Procedures for antibacterial susceptibility testing of *Adenopus breviflorus* (also known as *Leganaria breviflora*) against multiple resistant bacteria isolate



Flow Chart 2: Procedure for plasmid profiling analysis of multiple resistant *Staphylococcus aureus* using *Adenopus breviflorus* (also known as *Leganaria breviflora*)

Materials and Methods

Plant sample

Plant collection/source of *Adenopus breviflorus (Benth)*

The whole fruit of the Adenopus breviflorus (Benth) plants were obtained from a location named Akowo-Apete, Ibadan, Oyo state in the southwestern part of Nigeria and also gotten from the tropical rainforest of Ikare Akoko, Ondo state at exactly 9;00a m and 12;00am respectively.

Authentication of *Adenopus breviflorus (Benth)*

The plants were authenticated by a certified botanist at the herbarium unit of Department of Plant science and Biotechnology, Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria.

Preparation of *Adenopus breviflorus (Benth)*

The whole fruit was washed thoroughly with distilled water, stored in air tight containers and kept at room temperature prior to use [28].

Extraction

Extraction solvents

The extraction solvents used were ethyl acetate and absolute ethanol

Preparation of *Adenopus breviflorus (Benth)* for extraction

All the whole fresh fruit plant obtained were first washed thoroughly with sterile distilled water, chopped and thinly diced into a cubed shape. 400 g of each fresh whole fruit material was weighed separately into conical flasks containing 1200 ml of ethanol.

The mixtures were initially shaken rigorously and left for 24 days. All mixtures were filtered using sterile Whatman filter papers and the filtrates were collected directly into sterile crucibles. Extracts were collected and concentrated into a greenish-brown syrupy mass under reduced pressure using rotary evaporator at 40^0C to obtain the ethanol fraction The extract syrup formed was left over a water bath for final concentration into solid paste which gave a percentage yield of 21.8%. The concentrate was later reconstituted with 20% dimethyl sulphoxide (DMSO). The stock extracts were kept in the refrigerator at 40^0C until experimental use [29].

Percentage yield of *Adenopus breviflorus (Benth)* extracts

The 400g of fresh whole fruit extract of *Adenopus breviflorus (Benth)* yielded 5.5g after extraction.

Standardization of *Adenopus breviflorus (Benth)* extract

Using aseptic condition, the extract was reconstituted by adding 1.2 g of each extract with 2.5 ml of dimethylsulphoxide (DMSO) and 7.5 ml of sterile distilled water making it 100mg/mL. For each extract, 3ml of distilled water is measured into three sterile bijou bottles. In bijou bottle B 3 ml from 100 mg/mL extract was added and in bijou bottle C 3 ml from 50 mg/mL extract was added and bijou bottle D 3 mL from 25 mg/mL extract was added. A is 100 mg/ml, B is 50 mg/ml, C is 25 mg/mL D is 12.5 mg/mL respectively using the C1V1= C2V2 formulary [30].

Microorganism (isolates) used for research work

Microbial isolates used in this practical work used were standard multidrug resistant strains of bacteria isolate. They include *Bacillus cereus, Staphylococcus aureus, Staphylococcus typhii, Escherichia coli, Proteus vulgaris, Salmonella epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi,* and *Salmonella gallimarum.*

Source of test microorganisms

These organisms were obtained from the stock culture in the laboratory of the Department of Microbiology, Adekunle Ajasin University, Akungba-Akoko, and Ondo State, Nigeria.

Standardization of test organisms

Slants of the various organisms were reconstituted at aseptic condition, using a sterile wire loop; approximately one isolated colony of each pure culture was transferred into 5ml of sterile nutrient broth and incubated for 24hours. After incubation, 0.1ml of the isolated colony was transferred into 9.9 ml of sterile distilled water contained in each test tube using a sterile needle and syringe, and then mixed properly. The liquid now serves as a source of inoculum containing approximately 106 cfu/ml of bacterial suspension [31].

Antibacterial susceptibility testing of *Adenopus breviflorus (Benth)* extracts

Antibacterial assay of *Adenopus breviflorus (Benth)* extracts was carried out by using agar well diffusion method or technique. All the test organisms were sub-cultured onto sterile Mueller Hinton Agar plates and incubated at 37°C for 18-24 h. five distinct colonies for each organism were inoculated onto sterile Mueller Hinton broth and incubated for 3-4 h. All innocula was standardized accordingly to match the 0.5 Mc Farland standards and this standard was used for all susceptibility tests. All the extracts were reconstituted accordingly into the following concentrations: 100, 50, 25 and 12.5 mg/ml; using the Dimethyl Sulphoxide (DMSO).The susceptibility testing was investigated by the agar well diffusion method. A 0.1mL of 1: 10,000 dilutions (equivalent to 10^6cfu/ml) of fresh overnight culture of the multidrug resistant isolates grown in Nutrient agar broth was seeded into 40ml of Mueller Hinton agar, and properly mixed in universal bottles. The mixture was aseptically poured into sterile Petri dishes and allowed to set before wells were bored into the agar medium. Using a sterile cork borer of 6 mm diameter, equidistant wells were made in the agar. The wells were carefully filled up with prepared solution of the extracts (2 mL per well) with concentrations between 100 to 12.5 mg/ml Chloramphenicol 2 mg/ml was used as the control experiment. The plates were allowed to stand on the laboratory bench for an hour, to allow pre-diffusion of the extracts before incubation at 37°C for 24 hours, after which the plates were observed for the zones of inhibition. The zones of inhibition were measured to the nearest millimeter (mm) using a standard transparent meter rule. All experiments were performed in replicates of three or triplicate [32].

Determination of minimum inhibitory concentrations (MICs) of the ethanolic extract of *Adenopus breviflorus (Benth)* on bacterial strains

The MICs of the potent fractions of the extracts against the test organisms was determined using the standard method of European Committee for Antimicrobial Susceptibility testing by agar dilution. With some modifications Two-fold dilution of the extract was prepared in sterile distilled water and 2 mL of different concentrations of the solution was added to 18 mL of pre-sterilized molten nutrient agar. 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 24h old standardized bacterial cultures. The plates were later incubated at 37°C for 48 h. The plates were subsequently examined for the presence or absence of growth. A minimum inhibitory concentration (MICs) was taken as the lowest concentration that inhibits or prevented the growth of the isolates or bacterial growth. Sterile agar medium plate without the extract served as control. The experiment was carried out in three replicates [33].

Determination of minimum bactericidal concentrations (MBCs) of the ethanolic extract of *Adenopus breviflorus (Benth)* on bacterial strains

Minimum bactericidal concentrations of the extract were determined in accordance with the method of with some modifications. Samples for the MBC were taken from line of streak on MIC plates without visible growth and then streaked onto extract-free freshly prepared nutrient agar medium plates. The plates were then incubated at 37°C for 24 hrs. The MBC was taken as the lowest concentration of the extract that did not allow any bacterial growth on the surface of the agar plates at the end of 24 h incubation period and also that indicated a bactericidal effect after incubation. [34]

Plasmid Analysis of Multiple Resistant *Staphylococcus aureus* using *Adenopus breviflorus (Benth)* extracts

Determination of Plasmid Profile of Multiple Resistant *Staphylococcus aureus*

Plasmid extraction was carried out based on the methods of Molina-Aja with little modification. A single bacterial colony was picked up and grown in 5.0 mL of Muller Hilton broth overnight in an Eppendorf tube and centrifuged at 10,000 rpm for 2 min. The cell pellets obtained were re-suspended in 150 µl EDTA–Tris buffer and vortexes to mix. This was followed by the addition of 175 ul of 2% Sodium Dodecyl Sulphate (SDS) and

175ul of 0.4N NaOH. The tube was mixed vigorously, 250 ul of cold 5M potassium acetate was added vigorously, the tube was centrifuged at 12,000 rpm for 5 min and the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and equal volume of cold isopropanol was added. After inverting gently, the mixture was immediately centrifuged at 12,000 rpm for 10 min and the DNA pellet was washed with 650 µl of cold (40°C) 70% ethanol by centrifuging at 12,000 rpm for 15 min. The supernatant was discarded and the pellet was dried for 30 min and re-suspended in 40 µl of sterile deionized water. [35]

Gel Electrophoresis of Multiple Resistant *Staphylococcus aureus*

Agarose Gel Electrophoresis was carried out by weighing 0.8g of agarose powder and 100 mL of 1X Tris Borate Buffer (TBE buffer) was added, the buffer was dissolved by boiling in a microwave oven and allowed to cool to about 60°C and then 10 ul of ethidium bromide was added and mixed by swirling. The agarose was then poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5 mm and was allowed to solidify for about 20 minutes and the comb was removed, the tray was then placed in the electrophoresis tank. This was followed by the addition of 1X TBE buffer; this was then poured into the tank ensuring that the buffer covered the surface of the gel. The sample 15 ul was mixed with 2 ul of the loading dye and was carefully loaded into the wells created by the combs (marker was loaded in line 1). Electrodes were connected to the power pack in such a way that the negative terminal is at the end where the sample was loaded; electrophoresis was run at 60-100 V until loading dye has migrated about three-quarter of gel. Electrodes were disconnected and gel was removed from the tank and visualized in UV- trans-illuminator. [36]

Procedure for Plasmid Curing of Multiple Resistant *Staphylococcus aureus*

This was carried out based on the method described by Akinjogunla and Enabulele with slight medication. Fifty micro liters (50 µl) of Sodium Doecyl Sulphate (SDS) (0.10 mg/mL) was added to 5 mL of Lysogeny broth (LB) followed by subsequent culture inoculation of resistant Staphylococcus aureus with plasmid into separate LB broth having SDS. These were then incubated at 37°C for 24 hrs in a shaker. After incubation, the cultures were swabbed in to the Mueller Hinton Agar (MHA) plates for confirmatory antibacterial assay. [37]

Post Curing Susceptibility Testing of Multiple Resistant *Staphylococcus aureus* using *Adenopus breviflorus (Benth)* extracts

After incubation, the standardized innocula of these bacteria were swabbed in to the Mueller Hinton Agar (MHA) plates and incubated at 37°C for 18hrs as a confirmatory antibacterial assay. The plates were examined and the diameters of the zones of inhibition measured to the nearest whole millimeter with a ruler. The sizes of the zone of inhibition were then juxtaposed with those obtained before curing [38].

Time-kill kinetics assay from multiple resistant *Staphylococcus aureus* using active fractions of *Adenopus breviflorus (Benth)* extracts

Time-kill kinetics of ethanol extracts of Adenopus breviflorus (Benth) was carried out following the procedure described by Tsuji et al. Concentrations equal to MIC, twice the MIC, and four times the MIC of the extracts were prepared. An inoculum size of 1.0×10^6 CFU/mL was added and incubated at 37°C. Aliquots of 1.0 mL of the medium were taken at time intervals of 0, 30min, 3, 6, and 18 h, and inoculated aseptically into 20 mL nutrient agar and incubated at 37°C for24 h. A control test was performed for the organisms without the extracts or reference antibiotic. The colony forming unit (CFU) of the organisms was determined. The procedure was performed in triplicate (three independent experiments) and a graph of the log CFU/mL was plotted against time [39].

Determination of potassium ions leakage from multiple resistant *Staphylococcus aureus* using active fractions of *Adenopus breviflorus (Benth)* extracts

The method of All wood, Hugo and Gale was used for this assay. Cells of *S. aureus* from 18 hr old nutrient broth culture were washed in0.09 w/v NaCl (normal saline). Washed suspension of *S. aureus* (approximately 108 cells) were treated with various concentrations of the fractions relative to MIC at various time intervals of 0, 30min, 3, 6, and 18 hrs. Each suspension was then centrifuged at 10,000rpm and supernatant collected was assayed for potassium ion using atomic absorption spectroscopy. Normal saline inoculated with the same quantity of inoculums was used as control [40].

Determination of Qualitative Secondary metabolites screening of *Adenopus breviflorus (Benth)* extracts

Preliminary test / Preparation test

Adenopus breviflorus (Benth) filtrates were prepared by boiling 20 g of the fresh plant in distilled water. The solution was filtered through a vacuum pump. The filtrates were used for the photochemical screening for flavonoids, tannins, saponins, and alkaloids, reducing sugars, anthraquinones and anthocyanosides [41].

(i) Test for Alkaloids

About 0.2gram was warmed with 2% of H_2SO_4 for two minutes, it was filtered and few drops of Dragendoff's reagent were added. Orange red precipitate indicates the present of Alkaloids [42].

(ii) Test for Tannins

One milliliter of the filtrate were mixed with 2m1 of FeC1, A dark green color indicated a positive test for the tannins [43].

(iii) Test for Saponins

One milliliter of the *Adenopus breviflorus (Benth)* filtrate were diluted with 2 ml of distilled water; the mixture were vigorously shaken and left to stand for 10min during which time,

the development of foam on the surface of the mixture lasting for more than 10mm, indicates the presence of saponins [44].

(iv)Test for Anthraquinones

One milliliter of the *Adenopus breviflorus (Benth)* filtrate was shaken with 10ml of benzene; the mixture was filtered and 5 ml of 10 % (v/v) ammonia were added, then shaken and observed. A pinkish solution indicates a positive test [45].

(v) Test for Anthocyanosides

One milliliter of the *Adenopus breviflorus (Benth)* filtrate was mixed with 5 m1 of dilute HCI; a pale pink color indicates the positive test [46].

(vi) Test for Flavonoids

One milliliter of *Adenopus breviflorus (Benth)* filtrate was mixed with 2 m1 of 10% lead acetate; a brownish precipitate indicated a positive test for the phenolic flavonoids. While for flavonoids, I m1 of the plant filtrate were mixed with 2m1 of dilute NaOH; a golden yellow color indicated the presence of flavonoids [47].

(vii) Test for Reducing Sugars

One milliliter of the *Adenopus breviflorus (Benth)* filtrate was mixed with Fehling A and Fehling B separately; a brown color with Fehling B and a green color with Fehling A indicate the presence of reducing sugars [48].

(viii) Test for Cyanogenic glycosides

This was carried out subjecting 0.5g of the *Adenopus breviflorus (Benth)* extract 10ml sterile water filtering and adding sodium picrate to the filtrate and heated to boil [49].

Determination of Quantitative Secondary metabolites Screening of *Adenopus breviflorus (Benth)* extracts

(i) Saponins

About 20grams each of dried *Adenopus breviflorus (Benth)* samples were ground and, put into a conical flask after which 100 ml of 20 % aqueous ethanol were added. The mixture was heated using a hot water bath. At about 55^{0}C, for 4 hour with continuous stirring, after which the mixture were filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 rnl of diethyl ether were added and then shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times. 60 rnl of n-butanol were added. The combined n-butanol extracts were washed twice with 10 m1 of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage of the starting material [50].

(ii) Flavonoids

About 10 g of the *Adenopus breviflorus (Benth)* sample were extracted repeatedly with 100 ml of 80% aqueous methanol, at room temperature. The whole solution was filtered through Whatman filter paper No 42. The filtrate were later transferred into a crucible and evaporated into dryness over a water bath; the dry content was weighed to a constant weigh [51].

(iii) Cardiac glucosides

Legal test and the killer-kiliani was adopted, 0.5g of the *Adenopus breviflorus (Benth)* extract were added to 2ml of acetic anhydrate plus H_2S0_4 [52]

(iv)Tannins

About 500 mg of the *Adenopus breviflorus (Benth)* sample were weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 hour on a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the marked level. Then, 5 ml of the filtrate was transferred into a test tube and mixed with 2 ml of 0.1 M FeCl in 0.1 M Hcl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 minutes. The tannins content was calculated using a standard curve of extract [53].

(v) Alkaloids

Five grams of the *Adenopus breviflorus (Benth)* sample were weighed into a 250 ml beaker and 200ml of 10% acetic acid in ethanol was then be added, the reaction mixture were covered and allowed to stand for 4 hour. This was filtered and the *Adenopus breviflorus (Benth)* extract will be concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until the precipitation is complete. The whole solution were allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and then filtered; the residue being the alkaloid, which was dried and weighed to a constant mass [54].

(vi) Phlobatannins

About 0.5grams of each *Adenopus breviflorus (Benth)* extracts were dissolved in distilled water and filtered. The filtrate was boiled in 2% HCl, red precipitate show the present of phlobatannins [55].

Statistical Analysis of Data

All experiments were carried out in triplicate. Data were analyzed using the Statistical Package for Socio Sciences (version 20) and where applicable the simple descriptive statistics was carried out. Results were then presented in suitable tables and charts for summarization and simplicity.

Results

The results of the research work were demonstrated and recorded in table, figure and graph respectively.

Percentage yield results of whole fruit extract *Adenopus breviflorus (Benth)*

(Table1) The whole fruit extract of *Adenopus breviflorus (Benth)* was soaked into ethanol and the extract collected was

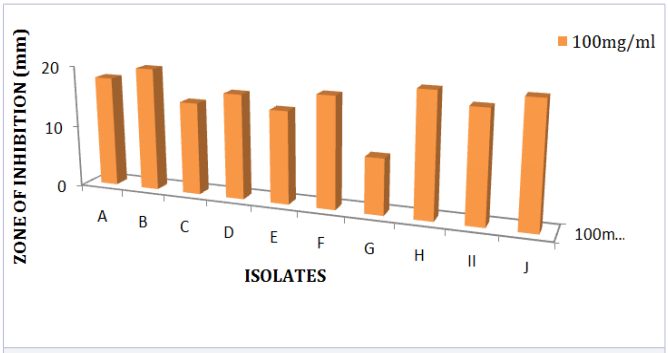
greenish brown in color. Table 1 showed the result for the yield extract of *Adenopus breviflorus (Benth)*, The whole fruit was used, The initial weight of the whole extract is 400 g, the volume of solvent used is 1,200 ml, The filtrate got was then left to air freeze while the residue of *Adenopus breviflorus (Benth)* ethanolic whole fruit extract is 8.2 g.

Table 1: Results for the yield of whole fruit *Adenopus eviflorus Benth)* extract

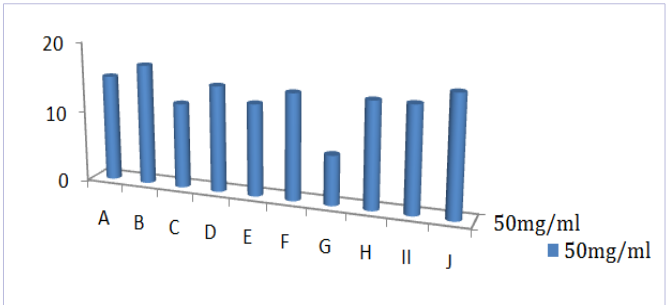
Plant part used	Initial weight	Volume of solvent	Ethanol
Whole fruit	400g	1,200ml	6.5g

Antimicrobial assay/ zones of inhibition results *Adenopus breviflorus (Benth)*

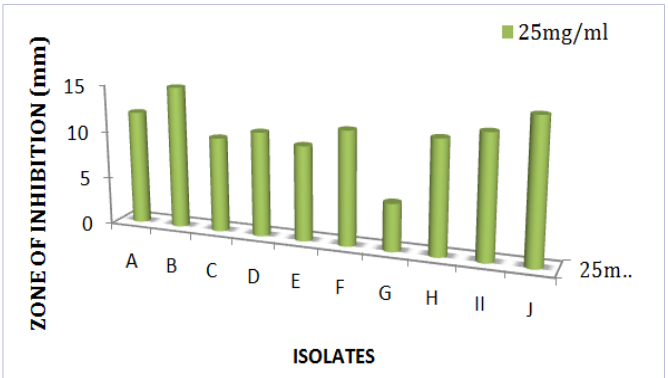
(Graph 1-4) (Antimicrobial assay).Shows the zones of inhibition of bacterial growth at different concentration (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) of ethanolic extract of *Adenopus breviflorus (Benth).* The antibacterial activities were expressed as the zone of inhibition diameters (mm) produced by the plant extract. The ethanolic extract of *Adenopus breviflorus* inhibited some of the bacteria with a measurable zone of inhibition.



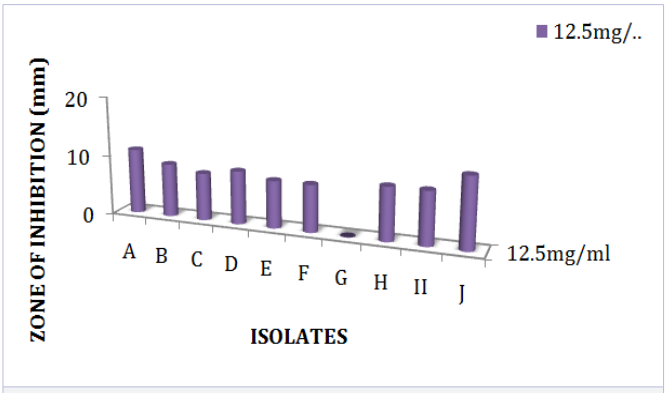
Graph 1: Antibacterial Screening of *Adenopus breviflorus (Benth)* Ethanolic Extract On Multiple drug Resistant Clinical Isolates At 100mg /ml Concentration



Graph 2: Antibacterial screening of *Adenopus breviflorus (Benth)* ethanolic extract against multiple drug resistant isolates at 50mg/ml concentration



Graph 3: Antibacterial screening of *Adenopus breviflorus (Benth)* ethanolic extract against multiple drug resistant isolates at 25mg/ml concentration.



Graph 4: Antibacterial screening of *Adenopus breviflorus (Benth)* ethanolic extract against multiple resistant isolates at 12.5mg/ml concentation.

The effects of the extract and chloramphenicol on *Salmonella gallinarium*

The ethanolic extract of *Adenopus breviflorus (Benth)* inhibited the growth of S. gallinarium colony. The zone of inhibition was dose dependent but the degree of inhibition recorded for each concentration of the extract declined with different concentrations. Comparatively the degree of inhibition for the extract (100mg/mL) was significantly lower than the inhibition recorded for chloramphenicol.

The effects of the extract and chloramphenicol on Bacillus cereus

Adenopus breviflorus (Benth) also inhibited the growth of B.cereus colony. The clear zone of inhibition was directly proportional to the dose administered. The area of inhibition produced by 100mg/ml of the extract was higher or significantly larger than the zone of inhibition caused by 12.5mg/ml of the extract. Comparatively the degree of inhibition caused by the extract (100mg/mL) was significantly lower than the inhibition recorded for chloramphenicol.

The effects of the extract and chloramphenicol on *Pseudomonas aeruginosa*

Adenopus breviflorus (Benth) roberty inhibited the growth of P.aeruginosa. The effects varied proportionally to the dose of extract administered on the culture plate. The bacteria colony was chloramphenicol sensitive and the zone of inhibition produced by chloramphenicol was significantly wider than that of the well treated with 100mg/ml of extract solution.

The effects of the extract and chloramphenicol on *Staphylococcus aureus*

Ethanolic extract of *Adenopus breviflorus (Benth)* inhibited the growth of S.aureus. The zone of inhibition produced by the 100mg/ml of the extract was significantly higher than inhibition produced by that of 12.5mg/ml of the extract.. Generally, zone of inhibition observed for the different doses of extract and chloramphenicol decreased with different concentration.

The effects of the extract and chloramphenicolon Escherichia coli

Ethanolic extract of *Adenopus breviflorus (Benth)* inhibited the growth of E. coli. The degree of inhibition was directly proportional to the dose administered. The degree of inhibition produced by the 100mg/mL of the extract was significantly higher than inhibition produced by 12.5 mg/mL of the extract. Comparatively, the degree of inhibition of the extract (100mg/mL) was slightly lower than the inhibition recorded for chloramphenicol. (Graph 1-4).

The effects of the extract and chloramphenicol on Proteus vulgaris

Ethanolic extract of *Adenopus breviflorus (Benth)* inhibited the growth of Proteus sp. The zone of inhibition was directly proportional to the dose of extract administered. The inhibition produced by the 100mg/ml of the extract was significantly higher than inhibition produced by 12.5mg/ml of the extract. Comparatively, the degree of inhibition for the extract (100 mg/ml) was slightly lower than the inhibition recorded for chloramphenicol (Graph 1-4).

The effects of the extract and chloramphenicol on *Klebsiella pneumonae*

Ethanolic extract of *Adenopus breviflorus (Benth)* inhibited the growth of Klebsiella sp. The zone of inhibition was directly to the dose to the dose of extract administered. The inhibition produced by the 100 mg/mL of the extract was significantly higher than the inhibition produced by 12.5 mg/mL of the extract. Comparatively the degree of inhibition for the extract was significantly lower than the inhibition recorded for chloramphenicol (Graph 1-4).

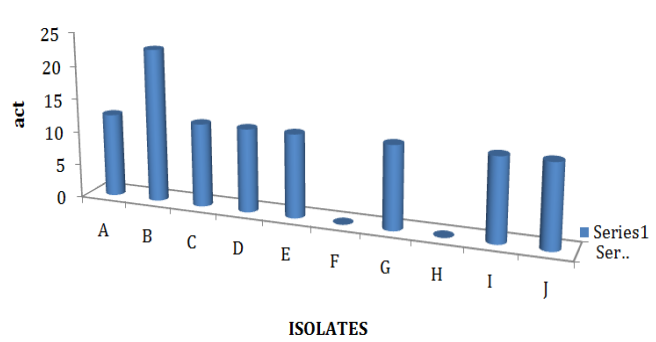
The effects of the extract and chloramphenicol on Staphylococcus epidermydis

Comparatively bacterial sensitivity for extract of *Adenopus breviflorus (Benth)*

Using the zone of inhibition of bacterial colonies to the antibacterial effects of *Adenopus breviflorus (Benth)*, the sensitivity of the bacterial colonies was observed in descending order as follows;

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results

(Graph 5) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Adenopus breviflorus (Benth)* Breviflora roberty Extract had antimicrobial activity against multiple drug resistant clinical isolates with minimum inhibitory concentration (MIC) ranging from 12.5to 25 mg/mL, The MBC of extract of *L. breviflora roberty* against test Gram-negative and Gram-positive bacteria were between the ranges of 25 to 50 mg/mL, MBC/MIC Ratios of Extracts: MBC/MIC ratios of extract of *Lagenaria breviflora roberty* against test Gram-negative and Gram-positive bacteria were between the ranges of 0.16 to 2.



Graph 5: Minimum inhibitory concentration of *Adenopus breviflorus (Benth)* extract against multiple drug resistant isolates

Qualitative and Quantitative secondary metabolite screening results

(Table 2) shows the qualitative secondary metabolite screening of *Adenopus breviflorus (Benth).* it was observed that Alkaloids, glycosides, steroids, Anthraquins, flavonoid. phenol, tannins, saponins, pyrrolizidine alkaloids, reducing sugar, terpenoid and cardiac glycoside were present in the whole fruit extract of *Adenopus breviflorus (benth)*. The volatile oil of the whole fruit was not present. The quantitative analysis of secondary metabolites screening of *Adenopus breviflorus (Benth)* using different solvents. This table shows the quantity in value of the secondary metabolites present in the whole fruit.

(Table 3) which is for methanol, shows that reducing sugar and alkaloids is the most abundant secondary metabolite in of *Adenopus breviflorus (Benth)* with 7.30 and others such as the alkaloid, glycoside, steroid, phenol, tannins, flavonoid, reducing sugar, terpenoid and cardiac glycoside had values ranging from 1.30 to 5.65 Volatile oil was not determined in the whole fruit of *Adenopus breviflorus (Benth).*

(Table 4) which is for ethanol, shows that alkaloids is the most abundant secondary metabolite in of *Adenopus breviflorus (Benth)* with value of 6.30 and others such as alkaloid, glycoside, steroid and pyrrolidizine alkaloid had values of 5.27, 5.68, 5.79, 2.33, 2.45, 2.25, 5.79 and volatile oil was not determined.

Table 2: Qualitative Analysis of Secondary metabolite screening of *Adenopus breviflorus (Benth) extract*

Sample	Alkaloid	Glycoside	Steroid	Anthraquin	Phenol	Tannins	Saponin	Flavonoi	Pyrrolizidine alkaloid	Reducing sugar	Terpenoid	Volatile oil	Cardiac glycosides
Leganaria breuflora	+ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve

Table 3: Quantitative Analysis of Secondary metabolite screening of *Adenopus breviflorus (Benth) extract*

Sample	Alkaloid	Glycoside	Steroid	Anthraquin	Phenol	Tannins	Saponin	Flavonoi	Pyrrolizidine alkaloid	Reducing sugar	Terpenoid	Volatile oil	Cardiac glycosides
Leganaria breuflora	7.30	7.27	5.65	5.71	2.36	2.42	1.25	1.30	1.87	7.30	7.27	ND	5.71

Table 4: Quantitative Analysis of Secondary metabolite screening of *Adenopus breviflorus (Benth) extract* ethanolic extract.

Sample	Alkaloid	Glycoside	Steroid	Anthraquin	Phenol	Tannins	Saponin	Flavonoi	Pyrrolizidine alkaloid	Reducing sugar	Terpenoid	Volatile oil	Cardiac glycosides
Leganaria breuflora	6.30	5.27	5.68	5.79	2.33	2.45	2.25	6.30	5.27	5.68	5.79	ND	2.45

Table 5: Quantitative Analysis of Secondary metabolite Screening of *Adenopus breviflorus (Benth) extract* ethyl acetate extract

Sample	Alkaloid	Glycoside	Steroid	Anthraquin	Phenol	Tannins	Saponin	Flavonoi	Pyrrolizidine alkaloid	Reducing sugar	Terpenoid	Volatile oil	Cardiac glycosides
Leganaria breuflora	4.62	5.24	9.78	0.10	0.32	1.14	1.20	4.62	0.24	9.78	0.10	ND	1.14

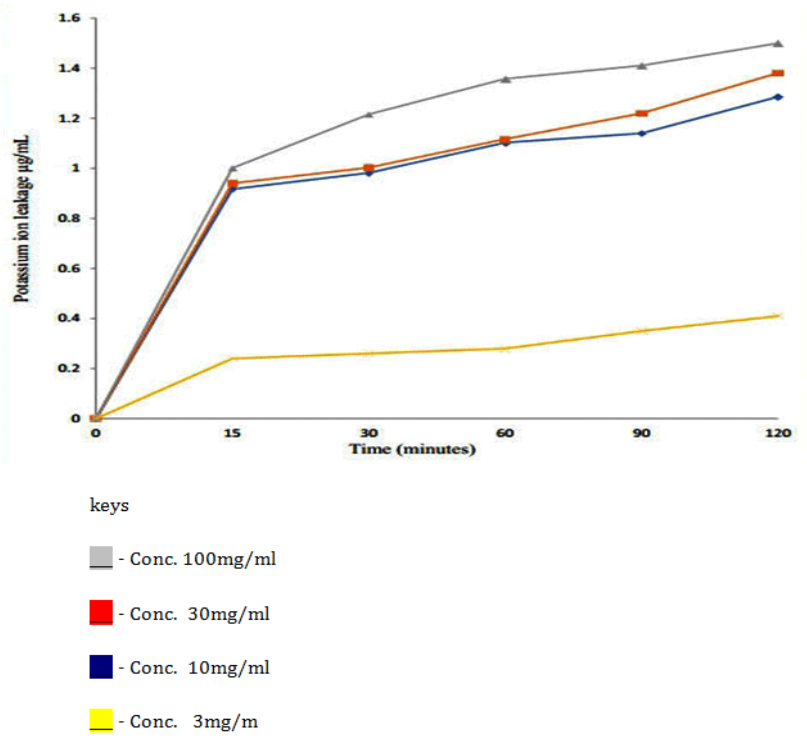
(Table 5) which is for ethyl acetate, shows that steroid and reducing sugar are the most abundant secondary metabolites with highest value of 9.78 for the *Adenopus breviflorus (Benth) extract* and other such as phenol, tannins, and cardiac glycoside had high values of 0.10 to 4.62 and the volatile oil for whole fruit was not determined.

Killing- kinetics results

Potassium efflux of *Adenopus breviflorus (Benth)* ethanolic extracts against multiple drug resistant *Staphylococcus aureus*

The potassium efflux of multiple drug resistant ***Staphylococcus aureus (MDRSA)*** treated with different concentrations of ethanolic extract of *Adenopus breviflorus (Benth)* in different time ranges. showed reduction in number of viable cells over the first, 30mins, 1hr, 3hr and 18hrs respectively, followed by a gradual rise up to the 24th h for *Staphylococcus aureus (MDRSA)* and when compared to the control (organisms without antimicrobial agent) The course of antimicrobial action was however observed to be bacteriostatic and concentration dependent for extracts of *Adenopus breviflorus (Benth)* studied. The area of the curve for *Adenopus breviflorus (Benth)* against *Staphylococcus aureus* at concentrations studied revealed that the number of cells was significantly (< 0.0001) reduced when compared to the control respectively.

(Graph 6) The graph below shows the potassium efflux of multi-drug resistant *Staphylococcus aureus* treated with different concentration of ethanolic extract of *Adenopus breviflorus (Benth)* in different time ranges.

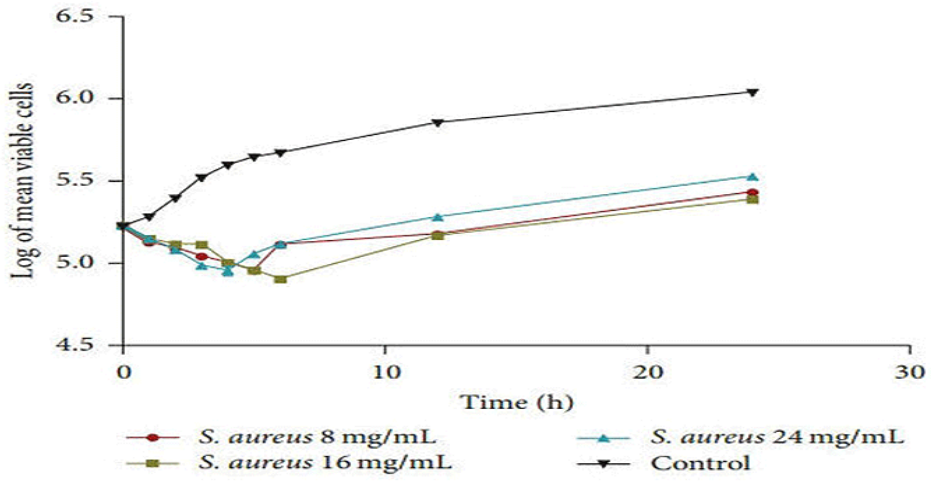


Graph 6: The graph below shows the potassium efflux of multi-drug resistant *Staphylococcus aureus* treated with different concentration of ethanolic extract of *Adenopus breviflorus (Benth)* in different time ranges.

Time-Kill Kinetics of *Adenopus breviflorus (Benth)* ethanolic extract against multiple drug resistant *Staphylococcus aureus*

The time-kill kinetics profile of multiple drug resistant *Staphylococcus aureus(MDRSA)* treated with different concentration of ethanolic extract of *Adenopus breviflorus (Benth)* in different time ranges. Showed reduction in number of viable cells over the first 3, 30mins, 3hr and 18hrs respectively, followed by a gradual rise up to the 24th h for Staphylococcus aureus and when compared to the control (organisms without antimicrobial agent). The course of antimicrobial action was however observed to be bacteriostatic and concentration dependent for extracts of *Adenopus breviflorus (Benth)* studied. The area of the curve for *Adenopus breviflorus (Benth)* against *Staphylococcus aureus* at concentrations studied revealed that the number of cells was significantly (< 0.0001) reduced when compared to the control respectively

(Graph 7) The graph below shows the microbial killing kinetic of different concentrations of ethanolic extract of *Adenopus breviflorus (Benth)* on multi-drug resistant *Staphylococcus aureus* in different time ranges.



Graph 7: The graph below shows the microbial killing kinetic of different concentrations of ethanolic extract of *Adenopus breviflorus (Benth)* on multi-drug resistant *Staphylococcus aureus (MDRSA)* in different time ranges.

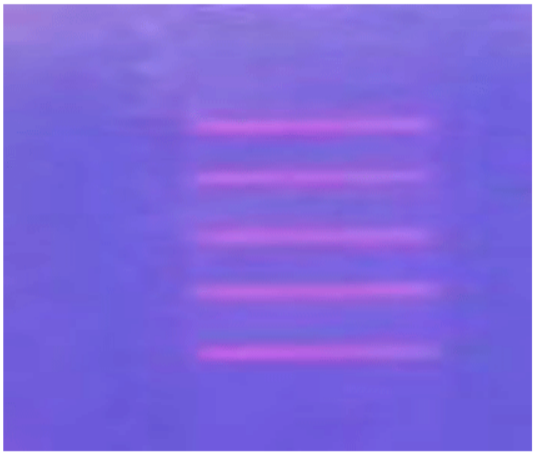


Figure 2: Pre-curing plasmid profile analysis of *Staphylococcus aureus(MDRSA).*

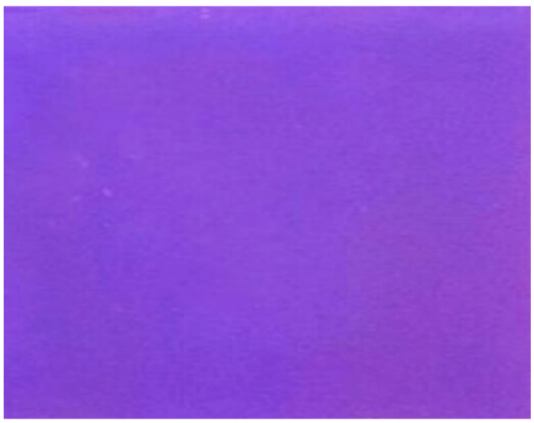


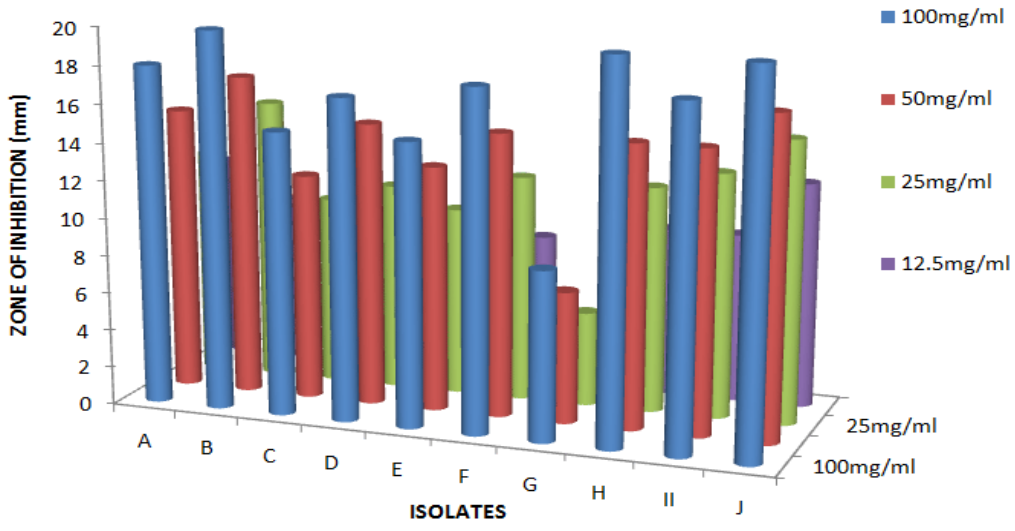
Figure 3: Post-curing plasmid profile of *Staphylococcus aureus(MDRSA).*

Pre and post plasmid curing result

(Figure 2 and Figure 3)

Discussion

This research has been able to investigate, identify and the efficacy of *Adenopus breviflorus (Benth)* on the sensitivity patterns of *Staphylococcus aureus(MDRSA)*, plasmid profile analysis and curing of multidrug-resistant bacteria using Staphylococcus aureus as a case study. *Adenopus breviflorus (Benth)* has antimicrobial activity on the multiple resistance isolates after which the Staphylococcus aureus was subject to plasmid curing. The multiple resistance clinical isolates was susceptible to extract of *Adenopus breviflorus (Benth)*(also known as Lagenaria breviflora Robert. This Medicinal plants are considered as a potential source of new chemotherapeutic drugs because of their diverse secondary metabolite (phyto chemicals) (present in abundant quantity [56, 57, 58] (Graph 8)



key

A; Bacillus cereus	***B; Klebsiella pneumonia***
C; Staphylococcus typhi	***D; Staphylococcus aureus***
E; Pseudomonas aeruginosa	***F; Staphylococus epidermydis***
G; Salmonella typhii	***H; Escherichia coli ;***
I; Proteus vulgaris	***J; Salmonella gallimarium***

Graph 8: Antibacterial Screening of *Adenopus breviflorus (Benth)*Ethanolic Extract On Multiple Resistant Isolates at Different Concentration

In this research work, antibacterial activities of the *Adenopus breviflorus (Benth)* extracts against the plasmid-carrying MDR bacterial strains were carried out at 12.5 to 100mcg/ml concentration. The possession of plasmids may have acquired their resistance through selective pressure from increased use and misuse of antimicrobial agents. The acquisition of resistance may due to chromosomal mutations or through plasmids that are capable of transfer from one strain of organism to another even across the species in addition to environmental influence. Thus, the gene coding for antibiotics resistance may either coded on the plasmid and chromosomal DNA. [71]. If plasmid can be remove through mechanism like disrupting plasmid replication by integrating into the DNA (e.g. intercalating agents and chlorpromazine), causing breaks in DNA and DNA (e.g. ascorbic acid) or by influencing plasmid super coiling (e.g. aminocoumarins and quinolones) and by preventing conjugation (e.g. unsaturated fatty acids and TraE inhibitors) [72]. Multi drug resistance bacteria will not be a problem in the microbial of infectious diseases. This is one of the focal point of this research work and it is clearly demonstrated this the result obtained during the course of the research work.

The ethanolic extract of *Adenopus breviflorus (Benth)* at 100 mg/ml was found to be more active against strains of *Bacillus cereus, Klebsiella pneumonia, Staphylococcus typhii, Staphylococcus aureus*, and *Pseudomonas aeruginosa. Staphylococus epidermydis, Salmonella typhi, Escherichia coli, Proteus vulgaris, Salmonella gallimarium.* High zone of inhibition was recorded against Salmonella gallimarum with the a diameter zone of inhibition between 15 to 20mm. *Klebsiella pneumonia* 20mm at 100mg/ml; 17mm at 50 mcg/ml and 15mm at 25mcg/ml respectively. This result was consistent with previous studies conducted elsewhere

where increase in the antimicrobial activities of the plant extract against the MDR organisms were reported to be attributable to the increase in the concentration and the nature of the active components. [59, 60, 61]

Recently, several studies have reported antibacterial activities of extracts *Adenopus breviflorus (Benth)*. While these reports have effectively represented the antibacterial activity of *Adenopus breviflorus (Benth)* less quantified and clear information regarding dose-response parameters and endpoints such as MBC, and MIC/MBC has been provided. Generally, the MIC of *Adenopus breviflorus (Benth)* the evaluated in this study ranging from 12.5 to 25.0mcg/ml and MBC from 5.0 to 40.0 mcg/ml. The MIC values of the *Adenopus breviflorus (Benth)* were lower than the MBC value indicating that; these plants are bacteriostatic at lower concentration and bactericidal at higher concentration; an assertion that has been documented by several researcher [62, 63, 64]. Inactivity of some of extracts at lower concentration was observed in this research work. This was probably attributed to the presence of plasmid in most of the organisms used. Multi Drug Resistance plasmids could be acquired by susceptible bacteria during treatment with antibiotics that can induce and select for horizontal transfer [65, 66, 67].

The qualitative secondary metabolite screening of *Adenopus breviflorus (Benth)* revealed the presence of medicinally active constituent such as cardiac glycoside, steroids, phenol, tannins, saponin, flavonoids, pyrrolidizine alkaloid, anthraquinone and reducing sugar while volatile oil was not determined (Table 4 above), all of which have been implicated with antimicrobial properties. some of which have been previously associated with antibacterial activity as observed by [68, 69, 70]; observed in his work that these plants possesses tannins, phlobotannins, alkaloids, saponins. The quantitative secondary metabolites screening of *Adenopus breviflorus (Benth)* using methanol, ethanol and ethyl acetate, showed the presence of different secondary metabolites in different quantities. These Medicinal plants are considered as a potential source of new chemotherapeutic drugs because of their diverse secondary metabolite (phytochemicals) (present in abundant quantity [56, 57, 58].

Staphylococcus aureus (MDRSA) that showed resistant before plasmid curing became sensitive to the antimicrobial plant extract of *Adenopus breviflorus (Benth)* after curing with Sodium Doecyl Sulphate (SDS) [73, 74]. This showed that the resistance is plasmid borne not chromosomal. Resistance to bacterial organisms not due to plasmid or chromosome might be due to efflux pump mechanism or other factors like mutation of gene encoding ribosomal protein which decrease permeability of the cell envelope in bacteria The screening of the bacterial isolates with SDS resultantly suggest that the resistance markers were stably lost [75, 76].

The basic concept of the time- kill kinetic study is establishment of the rate at which a choice microorganism is killed by the product which can either by the choice extracts or antibiotics as a function of survival instinct recorded at any exposure time period. Such data and graph can be constructed. The decline in population over time to a point of extinction the time kill analysis can also be monitored on the effect of various concentrations of an antimicrobial agent or extracts over time in relation to the stages of growth of choiced microorganism (Log, Lag and death phase) time kill kinetics assays [76].

The profiles of killing and re-growth of the *Staphylococcus aureus (MDRSA)* were measured over a course of 6 hours post inoculation. Time-kill studies are important because comprehensive information about pharmacodynamics from *Adenopus breviflorus (Benth)* extract antibacterial activity may not be gained simply through endpoints such as MIC [74]. Therefore, time-kill assays are required to quantitative pharmacodynamics of a plant antibacterial agent from by quantifying the decrease in bacterial growth as a function of time and the drug concentration The time-kill findings in this study displayed levels of time dependent bacterial inhibition in *Staphylococcus aureus (MDRSA)* and the concentrations, regardless of being gram-negative or gram-positive. It can be deduced that These findings might suggest that kinetics of responding of bacterial strains to the *Adenopus breviflorus (Benth)* extract during the first 6 hours of incubation does not necessarily depend to being gram-negative or gram-positive.

Conclusion

Development of bacterial resistance to multiple antibiotics has made treatment of infection diseases increasingly difficult over the past few years. Many known antibiotics have now become ineffective owing to the development and spread of plasmid encoded high level of resistance in bacteria. However, such ineffective antibiotics can be rendered effective if R-plasmids encoding antibiotic resistance are eliminated from the bacterial population. Unfortunately, plasmid curing agent can cure only a limited number of plasmids from a limited range of hosts. Hence, there is a continuing need of finding newer plasmid curing agents who are effective and less toxic. *Adenopus breviflorus (Benth) extract* showing plasmid curing properties is a needful medicinal plant for this purpose of removing the so-call multiple resistance saga in infectious diseases, In other words, the use of medicinal plant in the treatment of resistance bacteria causing infection must be encouraged and further research like isolation, extraction and purification of the bioactive component of this medicinal plant "*Adenopus breviflorus (Benth)*' should be further researched.

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