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# EVALUATION OF ANTIBACTERIAL, PHYTOCHEMICAL SCREENING AND GCMS PROFILE OF *Paullinia pinnata* Linn. LEAF EXTRACTS AGAINST CLINICAL WOUND ISOLATES

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## ABSTRACT

This study investigated the antibacterial properties, phytochemical constituents, and mode of action of *Paullinia pinnata* extracts on clinical wound isolates, and spectral features of the most active fraction. The *Paullinia pinnata* leaf was subjected to ethanol extraction and resulting solvent extract' concentrated to crude extract (Pp CE) using rotary evaporator at 4 - 40°C followed by fractionation with column chromatography. The antimicrobial effects of the extracts were determined against the test organisms using agar diffusion methods. Investigation of phytochemical constituents of *Paullinia pinnata* crude extract (Pp CE) and *Paullinia pinnata* ethyl acetate fraction (Pp EA) were carried out using appropriate methods. The constituents of the plant were further determined using GC-MS and FT-IR. The data obtained showed that the antibacterial activity of crude extract Pp CE, at a concentration of 35 mg/ml, had the highest zone of inhibition (22.00 mm) against *Staphylococcus* sp. (C1). The MIC and MBC recorded were 2.19 and 17.50 mg/mL respectively. Phytochemical screening of Pp EA detected alkaloid, terpenoid, saponin, phenol, carbohydrate, and resin as found in the Pp CE, but with additional cardiac glycoside and sterols. The Pp CE fraction attained appreciable rate of kill against representative strains, likely achieved by the antibacterial compounds present in the plant extract. The GC-MS revealed trans-13-Octadecenoic acid as the major constituent of Pp EA fraction at 23.25%. The FT-IR of the Pp EA fraction unveiled hydroxyl group, (C=O) carbonyl group of ketone/aldehydes and C-C skeletal vibrations. Thus, this study suggests that the Pp EA fractions possess some antibacterial activities and can be considered for development into therapeutic drugs.

**Keywords:** Phytochemical constituents; Ethyl acetate fraction; *Paullinia pinnata*; FT-IR; GC-MS.

## INTRODUCTION

Plants are abundant in secondary metabolites commonly used as medicinal and pharmaceutical interventions, the study of which is called phytochemistry. These chemical substances possess active constituents classified as secondary metabolites produced by plants and they contain therapeutic and pharmacological properties (Egbuna *et al.*, 2019). The field of pharmacotherapy investigates these substances considered as safer alternatives for effective disease management (Thummel and Shen, 2001).

One of such plants to be considered in this study, is a member of the Family Sapindaceae called *Paullinia cupana* of genus Paullinia which is a typical source of Guarana (a caffeine-rich (4 - 8 %) paste) produced from its seeds. This medicinal plant grows worldwide, thriving in Brazil and also, growing naturally in Madagascar, Jamaica, Domingo and South Africa. In other African countries like Zimbabwe and Zambia, it towers at an altitude of 1200 m and remain evergreen with other forest vegetation (Zamble *et al.*, 2006). The local Nigerian names of this plant include: Yoruba; Kakansenlaor Ogbe-okuje, Edo; Aza, Igala; Egwubi, Omekpa, Nupe; EnuKakanchela, and Hausa; Goorondoorinaa (Burkill, 2000).

The plant can be described as a climbing shrub that presents with compound leaves and winged rachis, its inflorescences stand axillary on long stalks bearing paired tendrils with white flowers. Its root, leaves, stem and entire plant have been used in various applications especially as caffeine, but very few investigations have been carried out on its other uses. Some studies have shown that the methanolic components of the root and leaf extracts are rich in phenols but there is paucity of information on its alkaloids components (Zamble

*et al.*, 2006).

Wounds infected with pathogenic microorganisms are the most common public health problems (Odimegwu *et al.,* 2008). These pathogens include bacteria, fungi, protozoa and viruses with the β-haemolytic Streptococci representing the most prevalent strains (i.e., *Streptococcus pyogenes*, *Staphylococcus aureus, Pseudomonas aeruginosa*), *Proteus* sp., *Escherichia coli* and *Enterococcus*, *Acinetobacter* sp., *Klebsiella* sp. (Mulu *et al.,* 2006; Rajan *et al.,* 2010; Mehta *et al.,* 2010) together with coliforms (Ozumba, 2007). The body's natural healing processes can quickly regenerate the dermal and epidermal tissues of such wound sites, but the chronic ones can adversely impact human health and result in decline of human economic capacity (Ousey and Mclntosh, 2009). Therefore, the aim of this study is to assess the phytochemical constituents of *P. pinnata* leaf against wound bacterial isolates which could proffer cheaper herbal-based alternatives for therapeutic management of wound infections.

Plate 1: *Paullinia pinnata* vine with its leaves and fruits (Source: Teaching and Research farm, Obafemi Awolowo University, Ile-Ife, Osun State

## MATERIALS AND METHODS

### Source of plant material and identification

Fresh and mature leaves of *Paullinia pinnata* used for this research were collected from the Teaching and Research Farm of Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria on the 22nd of March 2017 with latitude (7° 51 N), longitude (3° 56 E) and altitude 304 m. Voucher specimens with number FPI 2211 was thereafter allocated.

### Source of microorganisms

The test bacteria used for the study were clinical bacteria termed 'locally isolated organisms (LIO)', all isolated from human infected wounds obtained from Department of Microbiology and Parasitology, Obafemi Awolowo University Teaching Hospital, Ile-Ife, Osun State. They were subjected to various conventional biochemical tests for identification as follows: Gram-positive bacteria were *Staphylococcus aureus*, *Staphylococcus* sp. (C1), *Staphylococcus* sp. (C2), *Streptococcus canis* (LIO), *Staphylococcus massiliensis* (LIO), *Streptococcus pyogenes* (LIO), *Streptococcus* sp. (C3), *Streptococcus* sp. (C4), *Macrococcus* sp. (C5), *Macrococcus* sp. (C6), *Globicatella* sp. (C7) *and Salinicoccus* sp. (C8). The Gram-negative bacteria included: *Klebsiella pneumoniae* (LIO), *Klebsiella ozanae* (LIO), *Enterobacter asburiae* (LIO), *Aerococcus suis* (LIO), *Salmonella enterica* (LIO), *Liminorella* sp. (C9), *Citrobacter* sp. (C10), *Liminorella* sp. (C11), *Citrobacter* sp. (C12), *Proteus* sp. (C13), *Yersinia* sp. (C14) and *Proteus vulgaris* (LIO).

### Culture media

All the culture media such as: Nutrient broth, Nutrient agar, Mueller-Hinton agar used in this study were manufactured by Lab M Ltd, UK. Nutrient broth and Nutrient agar were used for culture of bacterial isolates, Mueller-Hinton agar was for determination of antibacterial activity of the plant leaf extract and Nutrient Agar was used to assess the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the plant leaf extracts on the test bacteria. The locally isolated organisms (LIO) were subsequently stored on Nutrient agar slants at 4°C. All media used were prepared and sterilized at 121°C for 15 min.

**Drying and extraction of the plant material**

The leaves of *Paullinia pinnata* were collected, air-dried under the shade at room temperature (25±1 °C) to constant weight and subsequently ground into powder. To prepare a standard concentration of 35 mg/mL, a weight of 175 mg crude leaf extract of *Paullinia pinnata* was dissolved in 5 mL of DMSO/$H_2O$ (2:3). Further dilutions (17.5 mg/mL, 8.75 mg/mL, 4.38 mg/mL, 2.19 mg/mL) were prepared from the standard stock solution for other experiments.

**Column chromatography of the leaf extract of *Paullinia pinnata***

The column chromatography method was employed where silica gel was the stationary phase and various solvents (*n*-hexane, ethyl acetate, acetone, and ethanol) were the mobile phases in order of increasing polarity. To start with, a weight of 62.6 g of the crude leaf extract was adsorbed unto silica gel (separating layer) using minimum amount of ethyl acetate. Thereafter, elution with organic solvents was performed based on their order of polarity as follows: n-hexane, ethyl acetate, acetone and ethanol. The resulting fractions of each solvent were collected in clean conical flasks and concentrated to dryness using rotary evaporator at 40 °C. To assess the synergistic effect of fractions of *Paullinia pinnata* leaf extract, the ethyl acetate and n-hexane fractions were used in combination of ratio 1:1 (w/w).

**Qualitative phytochemical screening test of the extract**

The preliminary qualitative phytochemical analysis of the crude leaf extract of *Paullinia pinnata* was carried out to screen for the bio-active compounds resident in the leaf sample.

**Determination of the minimum bactericidal concentrations (MBCs) of ampicillin, Pp CE and Pp EA fractions on wound bacterial isolates.**

The Minimum Inhibitory Concentration (MIC) of ampicillin, crude extract (Pp CE), and the fractions obtained were determined according to (NCCLS, 2002) with some modifications. Freshly prepared Nutrient Agar plates were inoculated with 18-24 h cultures, incubated at 37 ºC for 24 h. Also, the least concentration at which no growth appeared was recorded as Minimum Bactericidal Concentration (MBC).

**The extent of rate of killing exhibited by ampicillin, crude extract (Pp CE) and active fractions against *P vulgaris* and *S. aureus***

The rate of kill assay was carried out using the method described by Odenholt *et al.* (2001) with some modifications. The ampicillin, Pp CE and other active fractions were tested against representative Gram-positive *Proteus vulgaris* and Gram-negative *Staphylococcus aureus* with their resulting viable counts determined. For each test organism, an aliquot of 0.5 mL of known cell density (by viable counts $10^8$ cfu/mL) was added to 4.5 mL of different concentrations of the crude extract, ampicillin and active fractions.

The suspension was thoroughly mixed, held at room temperature (28-30 ºC) and the time-kill kinetics determined at time intervals of 0, 15, 30, 60, 90 and 120 min. A volume of 0.5 mL of the suspension was withdrawn at these time intervals and transferred to 4.5 mL nutrient broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compounds from the test suspensions.

The suspension was again mixed thoroughly and serially diluted up till $10^{-7}$ in sterile physiological saline with subsequent transfer of 0.5 mL of this final dilution into pre-sterile molten nutrient agar at 45 ºC followed by plating and incubation at 37

ºC for 72 h. Control experiment was set up to exclude the antimicrobial agent. Experiments were done in duplicates and decrease in the viable counts with time indicated actual killing of the representative strains by the antimicrobial agents.

**Determination of potassium ions leakage by ethyl acetate fraction (Pp EA) on the test bacteria cells**

To determine the leakage of potassium ions from the representative organisms, method of Lee *et al.* (2002), was used for this assay since increase in $K^+$ efflux will indicate cell membrane damage. The 18-h old broth culture (approximately $10^8$ inoculum, 0.5 McFarland standard) of *Proteus vulgaris* and *S. aureus* were washed in 0.09 w/v NaCl (normal saline) and then, treated with various concentrations of the active fractions at previously used time intervals for 2 h (according to pre-determined MIC values). After this, the preparations were centrifuged at 10,000 rpm and supernatant assayed for potassium ($K^+$) ion using atomic absorption spectroscopy. A similar set-up with normal saline inoculated with the standardized inoculum was used as control.

**Determination of nucleotide leakage by ethyl acetate fraction (Pp EA) on the test bacteria cells**

The nucleotide leakage of *P. vulgaris* and *S. aureus* cells was determined according to method by Akinpelu *et al.* (2015b), with some modifications. The 18-h old nutrient broth culture (approximately $10^8$ inoculum, 0.5 McFarland standard) were washed in 0.9% (w/v) normal saline and treated with various concentrations of the active fractions at previously used time intervals for 2 h (according to pre-determined MIC values). Each preparation was centrifuged at 10,000 rpm and optical density of the supernatant measured at wavelength of 260 nm. A similar set-up with normal saline inoculated with the standardized inoculum was used as control.

**Determination of protein leakage by ethyl acetate fraction of *P. pinnata* (Pp EA) on the test bacteria cells**

The leakage of protein from *Proteus vulgaris* and *S. aureus cells* were determined by preparing 18 h broth cultures (approximately $10^8$ inoculum, 0.5 McFarland standard) of both organisms and washed in 0.09 w/v NaCl (normal saline). Thereafter, they were treated with various concentrations of the Pp EA fractions at previously used time intervals for 2 h (according to pre-determined MIC values). Each suspension was then centrifuged at 7000 rpm and resulting supernatant assayed for protein content. A volume of 0.4 mL Bradford reagent was added to 1.6 mL of each sample and optical density (OD) of the resulting supernatant determined at wavelength of 595 nm after 5 min and protein value extrapolated from the Bovine Serum Albumin (BSA) standard curve.

Gas chromatography-mass spectrometry (GC-MS) of ethyl acetate fraction of *P. pinnata* (Pp EA)

Gas chromatography-Mass spectrophotometry (GC-MS) of the Pp EA fraction was performed using Agilent technologies GC system comprising on AOC-20i auto-sampler and a Gas chromatograph interfaced a triple axis mass spectrometer detector equipped with an Elite-5MS (5% diphenyl/95% dimethyl pol siloxane) fused to a capillary column (30 x 0.25 μm df). The mass-detector used in this analysis was Agilent Technologies-5975C while gas chromatography model was Agilent Technologies-7890A. The injector model used was Agilent Technologies-7683B, and the software adopted to handle mass spectra and chromatograms was a NIST version 14.0L. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.99%) was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 2 μL was employed (a split ratio of 10:1).

The injector temperature was maintained at 250 ºC, ion-source temperature was 200 ºC, oven temperature was programmed from 110α (isothermal for 2 min) with an increase of 10 ºC/min to 200 ºC, then 5 ºC/min to 280 ºC, ending with a 9 min isothermal at 280 ºC. Mass spectra were taken at 70 eV: a scan interval of 0.5 s. The solvent delay was 0 to 6 min, and the total GC-MS running time was 34.667 s. The relative amount (% each component) was calculated by comparing its average peak to the total areas (Lu, 2003).

Fourier transform infrared spectrophotometer

(FT-IR) of the Pp EA fraction

The FT-IR is typically used to detect diverse chemical bonds (functional groups) integral in chemical compounds. Each chemical bond will absorb a characteristic wavelength of light as observed in a resulting annotated spectrum and further interpretation of this absorption spectrum can elucidate the chemical bonds in a molecule. The powdered extracts of *Paullinia pinnata* are suitable for FT-IR analysis, thus, about 10 mg of the dried powder of ethyl acetate fraction was encapsulated in 100 mg of KBr pellet, to prepare translucent sample discs. This preparation was loaded in FT-IR spectroscope, with a scan ranged from 400 to 4000 $cm^{-1}$ and resolution of 4 $cm^{-1}$ (McMurry, 2000).

**Statistical analysis of data**

For the statistical analysis of data obtained in this study, the experiments were carried out in triplicates and results presented as mean standard deviation (SD). Student's t-test was used to compare two means and one-way analysis of variance (ANOVA) was used for comparison of more than two means.

## RESULTS

**Table 1**: Measuring the zones of inhibition (mm**) of crude ethanolic extract of *P. pinnata* Leaf against test wound bacterial isolates

| Test Bacteria | Ethanolic crude extracts of *P. pinnata* Leaf | | | Ampicillin |
|---|---|---|---|---|
| | 8.75 mg/mL | 17.5 mg/mL | 35 mg/mL | (1 mg/mL) |
| *Macrococcus* sp. (C6) | 0.00 | 0.00 | 5.00±1.41 | 25.00±1.22 |
| *Aerococcus suis* | 9.00±1.41 | 10.00±1.41 | 12.00±1.41 | 24.00±2.12 |
| *Staphylococcus aureus* | 8.00±1.41 | 12.00±1.41 | 15.00±1.41 | 20.00±1.50 |
| *Citrobacter* sp. (C12) | 12.00±0.71 | 11.00±1.41 | 15.00±0.71 | 21.00±0.00 |
| *Globicatella* sp. (C7) | 11.00±1.41 | 17.00±1.41 | 19.00±0.85 | 22.00±1.55 |
| *Streptococcus canis* | 5.00±1.41 | 7.00±1.41 | 11.00±1.41 | 25.00±1.88 |
| *Staphylococcus* sp. (C2) | 10.00±1.41 | 11.00±1.41 | 16.00±1.41 | 25.00±1.05 |
| *Citrobacter* sp. (C10) | 9.00±0.00 | 11.00±1.41 | 15.00±1.27 | 25.00±0.34 |
| *Proteus vulgaris* | 9.00±0.71 | 12.00±0.71 | 15.00±1.84 | 26.00±0.13 |
| *Staphylococcus massiliensis* | 0 | 13.00±0.00 | 18.00±1.41 | 19.00±0.71 |
| *Salmonella enterica* | 0 | 0 | 0 | 25.00±0.85 |
| *Staphylococcus* sp. (C1) | 10.00±0.14 | 14.00±0.14 | 20.00±1.41 | 24.00±1.27 |
| *Salinicoccus* sp. (C8) | 8.00±0.71 | 11.00±1.41 | 15.00±1.41 | 22.00±0.87 |
| *Streptococcus pyogenes* | 6.00±1.41 | 9.00±1.41 | 12.00±1.41 | 19.00±2.44 |
| *Streptococcus* sp. (C3) | 5.00±1.41 | 9.00±1.41 | 13.00±1.41 | 0 |
| *Liminorella* sp. (C9) | 6.00±1.41 | 10.00±1.41 | 12.00±1.88 | 17.00±2.44 |
| *Klebsiella ozanae* | 0 | 0 | 0 | 19.00±0.00 |
| *Streptococcus* sp. (C4) | 0 | 0 | 0 | 13.50±1.20 |
| *Klebsiella pneumoniae* | 0 | 0 | 0 | 18.00±2.22 |
| *Enterobacter asburia* | 0 | 0 | 0 | 0 |
| *Proteus* sp. (C13) | 0 | 0 | 0 | 0 |
| *Yersinia* sp. (C14) | 0 | 0 | 0 | 0 |
| *Liminorella* sp. (C11) | 0 | 0 | 0 | 0 |
| *Macrococcus* sp. (C5) | 7.00±1.88 | 15.00±1.41 | 18.00±1.41 | 30.00±1.32 |

**KEY:** All organisms are clinical isolates, locally isolated from human infected wound, ** = mean of three replicates, SD = Standard Deviation

C1 - *Staphylococcus* sp., C2 - *Staphylococcus* sp., C3 - *Streptococcus* sp., C4 - *Streptococcus* sp., C5 - *Macrococcus* sp., C6 - *Macrococcus* sp., C7 - *Globicatella* sp., C8 - *Salinicoccus* sp., C9 - *Liminorella* sp., C10 - *Citrobacter* sp., C11 - *Liminorella* sp., C12 - *Citrobacter* sp., C13 - *Proteus* sp., C14 - *Yersinia* sp.

**Table 2**: Antimicrobial activities exhibited by the *Paullinia pinnata* (mm**) Leaf fractions singly and synergistically against wound bacterial isolates

| Test bacteria | Ethanol (10 mg/mL) | Acetone (10 mg/mL) | Ethylacetate (10 mg/mL) | n-Hexane (10 mg/mL) | Ethylacetate/n-hexane (10 mg/mL) |
|---|---|---|---|---|---|
| *Proteus vulgaris* | 0 | 0 | 20.00±1.84 | 16.00±0.00 | 20.00±0.00 |
| *Staphylococcus massiliensiss* | 0 | 0 | 24.00±1.41 | 14.00±1.41 | 23.00± 1.21 |
| *Staphylococcus aureus* | 0 | 0 | 26.00±1.27 | 16.00±2.69 | 24.00±2.10 |
| *Aerococcus suis* | 0 | 0 | 28.00±1.27 | 22.00±1.41 | 26.50±0.68 |
| *Macrococcus* sp (C5) | 0 | 0 | 25.00±1.41 | 17.00±0.00 | 22.00±1.66 |
| *Liminorella* sp (C9) | 0 | 0 | 15.00±1.41 | 8.00±0.14 | 16.00±1.41 |
| *Staphylococcus* sp (C2) | 0 | 0 | 22.00±0.85 | 17.00±0.71 | 14.00±1.85 |
| *Staphylococcus* sp (C1) | 0 | 0 | 9.00±1.41 | 18.00±0.85 | 18.50±1.21 |
| *Streptococcus canis* | 0 | 0 | 0 | 8.00±1.88 | 13.00±1.14 |
| *Streptococcus pyogenes* | 0 | 0 | 0 | 17.00±1.41 | 24.00±1.85 |
| *Citrobacter* sp (C12) | 0 | 0 | 21.00±1.88 | 15.00±1.85 | 14.00±1.88 |
| *Streptococcus* sp (C3) | 0 | 0 | 9.00±1.41 | 0 | 18.00±1.22 |
| *Globicatella* sp (C7) | 0 | 0 | 25.00±1.85 | 18.00±1.21 | 17.50±1.41 |
| *Macrococcus* sp (C6) | 0 | 0 | 7.00±1.21 | 15.00±1.41 | 12.00±2.44 |
| *Citrobacter* sp (C10) | 0 | 0 | 10.00±1.22 | 6.00±1.88 | 20.00±1.22 |
| *Salinicoccus* sp (C8) | 0 | 0 | 22.00±1.41 | 15.00±1.85 | 22.00±1.88 |

**KEY:** ** = mean of three replicates, SD = Standard Deviation
C1 - *Staphylococcus* sp., C2 - *Staphylococcus* sp., C3 - *Streptococcus* sp., C4 - *Streptococcus* sp., C5 - *Macrococcus* sp., C6 - *Macrococcus* sp., C7 - *Globicatella* sp., C8 - *Salinicoccus* sp., C9 - *Liminorella* sp., C10 - *Citrobacter* sp., C11 - *Liminorella* sp., C12 - *Citrobacter* sp., C13 - *Proteus* sp., C14 - *Yersinia* sp.

**Table 3**: Phytochemical Constituents of the Pp CE and Pp EA fractions

| Test bacteria | Pp CE | Pp EA |
|---|---|---|
| Saponins | ++ | ++ |
| Tannins | - | - |
| Flavonoids | - | - |
| Terpenoids | ++ | ++ |
| Cardiac glycosides | ++ | - |
| Alkaloids | ++ | ++ |
| Resins | **++** | **++** |
| Phlobatannins | - | - |
| Sterols | ++ | - |
| Phenols | ++ | ++ |
| Carbohydrates | ++ | ++ |

**KEY:** ++ = present, - = absent

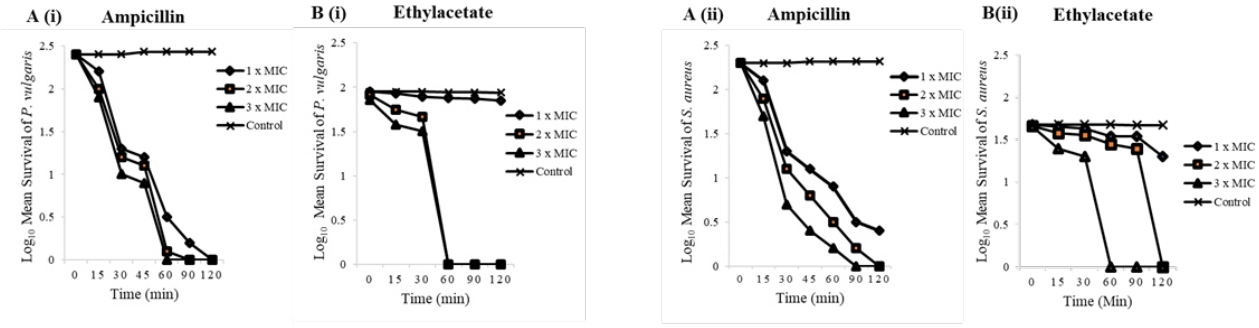


**Figure 2**: The extent and rate of kill of Ampicillin and Pp EA on *Proteus vulgaris* and *Staphylococcus aureus* at 1 x MIC, 2 x MIC and 3 x MIC concentrations (8.75 mg/mL, 17.50 mg/mL and 35.00 mg/mL respectively). (A & B) Effect of ampicillin (control) and ethyl acetate fractions on *P. vulgaris*. (C & D) Effect of ampicillin (control) and ethyl acetate fractions on *S. aureus*. The Pp EA fraction attained 100% killing rate similar to ampicillin (standard antibiotic) at 60 min for both *Proteus vulgaris* and *Staphylococcus aureus*.

**KEY:** Each point represents the $\log_{10}$ mean survival of bacterial cells at a particular time interval in the presence of the antibiotic.

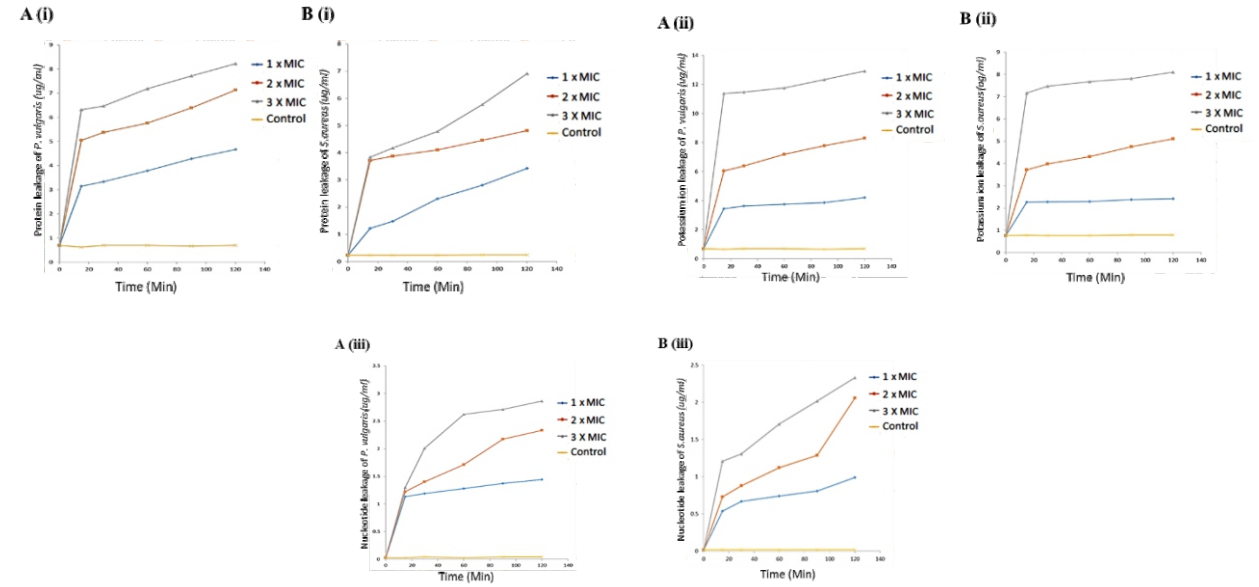


**Figure 3:** The effect of Pp EA fraction on nucleotide, potassium ion and protein leakage of *Proteus vulgaris* and *Staphylococcus aureus* at 1 x MIC, 2 x MIC and 3 x MIC concentrations A(i) Pp EA on nucleotide leakage attained highest concentration of 3 x MIC, 2.86 µg/mL on *P. vulgaris* and 3 x MIC, 2.33 µg/mL on *S. aureus* both at 120 min contact time. A(ii) Pp EA on potassium ion leakage attained highest concentration of 3 x MIC, 12.33 µg/mL on *P. vulgaris* and 3 x MIC, 8.10 µg/mLon *S. aureus* at 120 min contact time. A(iii) Pp EA on protein leakage attained highest concentration of 3 x MIC, 8.22 µg/mL on *P. vulgaris* and 3 x MIC, 6.91 µg/mL on *S. aureus* at 120 min contact time.

**Key:** Each point represents the mean $\log_{10}$ survival of bacterial cells at a particular time interval in the presence of the antibiotic.

**Table 4:** Relative abundance of major chemical consituents of Ethyl-acetate *P. pinnata* fraction

| S/N | Chemical constituents | Pp EA fraction (%) |
|---|---|---|
| 1 | 1,2-Benzenedicarboxylic acid | 5.70 |
| 2 | 4-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl]-2-cyclohexen-1-one) | 6.99 |
| 3 | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | 7.79 |
| 4 | 1,3,5-Triazine, hexahydro-1,3,5-tris (2,2,6,6-tetramethyl-4-piperidinyl) | 17.22 |
| 5 | Trans-13-Octadecenoic acid | 23.35 |

**Table 5:** Chemical consituents of ethyl acetate *P. pinnata* fraction (Mass Peak: 395,400)

| S/N | Name | Molecular Weight | Structural Formula | Ret. Index |
|---|---|---|---|---|
| 1. | Caprolactam | 113 | $C_6H_{11}NO$ | 1003 |
| 2. | 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxypheno | 180 | $C_{10}H_{12}O_3$ | 1653 |
| 3. | 2,3-Dimethyl-2-(3-oxobutyl) cyclohexanone | 196 | $C_{12}H_{20}O_2$ | 1521 |
| 4. | 5,5,8a-Trimethyldecalin-1-one | 194 | $C_{13}H_{22}O$ | 1481 |
| 5. | 4-Hydroxy-3,5,5-trimethyl-4-[3-oxo-1-butenyl]-2-cyclohexen-1-one | 222 | $C_{13}H_{18}O_3$ | 1751 |
| 6. | (4-Methoxy-phenyl) - (10-methyl-10H-acridin-9-ylidene)-amine | 314 | $C_{21}H_{18}N_2O$ | 2782 |
| 7. | Phytol acetate | 338 | $C_{22}H_{42}O_2$ | 2168 |
| 8. | 1,3,5-Triazine, hexahydro-1,3,5-tris (2,2,6,6-tetramethyl-4-piperidinyl | 504 | $C_{30}H_{60}N_6$ | 3985 |
| 9. | Isobutyl undec-2-en-1-yl ester | 374 | $C_{23}H_{34}O_4$ | 2676 |
| 10. | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | 296 | $C_{20}H_{40}O$ | 2045 |
| 11. | 1,2-Benzenedicarboxylic acid | 278 | $C_{16}H_{22}O_4$ | 1908 |
| 12. | 5-methyl-Octadecane | 268 | $C_{19}H_{40}$ | 1846 |
| 13. | 1-(+)-Ascorbic acid 2,6-dihexadecanoate | 652 | $C_{38}H_{68}O_8$ | 4765 |
| 14. | Phytol | 296 | $C_{20}H_{40}O$ | 2045 |
| 15. | 5-methyl-heneicosane | 310 | $C_{22}H_{46}$ | 2144 |
| 16. | 6,6-Diethylhoctadecane | 310 | $C_{22}H_{46}$ | 2124 |
| 17. | Trans-13-Octadecenoic acid | 282 | $C_{18}H_{34}O_2$ | 2175 |
| 18. | Eicosane | 282 | $C_{20}H_{42}$ | 2009 |
| 19. | Tetracosane | 338 | $C_{24}H_{50}$ | 2407 |
| 20. | 5,5-Diethylheptadecane | 296 | $C_{21}H_{44}$ | 2024 |
| 21. | Tetratetracontane | 618 | $C_{44}H_{90}$ | 4395 |
| 22. | Hexadecanoic acid | 330 | $C_{19}H_{38}O_4$ | 2498 |
| 23. | Diisooctyl phthalate | 390 | $C_{24}H_{38}O_4$ | 2704 |
| 24. | Hexatriacontane | 506 | $C_{36}H_{74}$ | 3600 |
| 25. | Tetratriacontane | 478 | $C_{34}H_{70}$ | 3401 |
| 26. | 2-methylhexacosane | 380 | $C_{27}H_{56}$ | 2641 |
| 27. | 2-methyloctacosane | 408 | $C_{29}H_{60}$ | 2840 |
| 28. | Cholesta-4,6-dien-3-ol | 384 | $C_{27}H_{44}O$ | 2579 |
| 29. | Stigmast-5-en-3-yl (9Z)-9-octadecenoate | 678 | $C_{47}H_{82}O_2$ | 4469 |
| 30. | Beta-Sitosterol | 414 | $C_{29}H_{50}O$ | 2731 |

**KEY:** Ret. Index - Retention Index

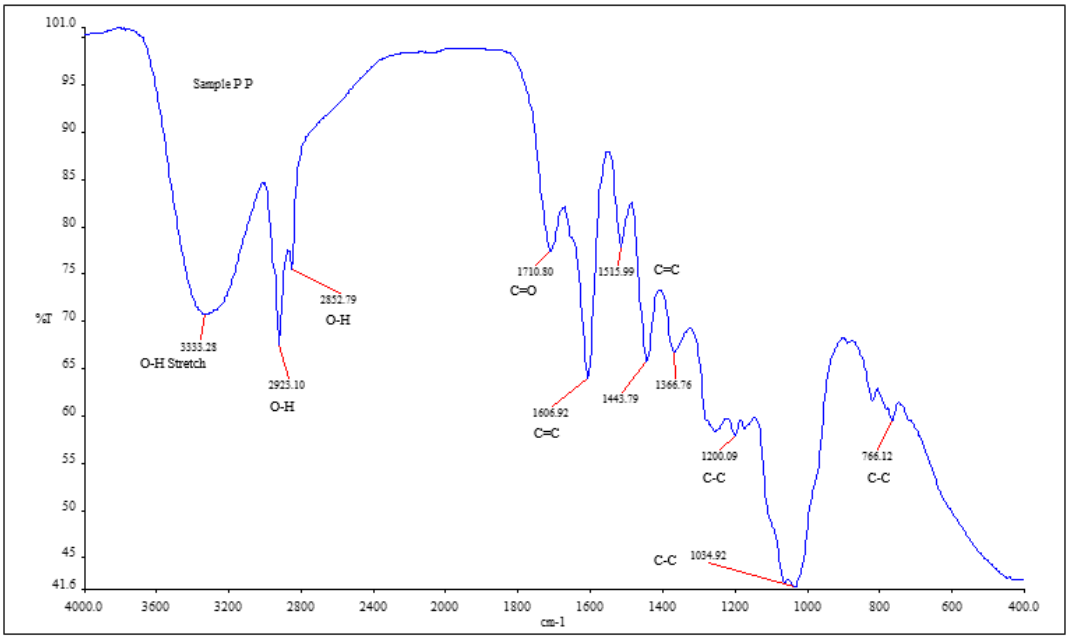


**Figure 4**: FT-IR Spectrum of the Pp EA (ethyl acetate) fraction of *Paullinia pinnata* showing organic ligands.

**Table 6:** FT-IR analysis of the Pp EA (ethyl acetate) fraction

| Absorption Bands ($cm^{-1}$) | Functional Group Represented |
|---|---|
| 3333 | Hydroxyl group, H-bonded OH Stretch |
| 2923/2853 | CH Stretch |
| 1711 | Carbonyl group of ketone/aldehyde |
| 1367 and 1516 | Aromatic ring stretch (C=C) |
| 1200, 1034 and 766 | Skeletal C=C vibration |

## DISCUSSION

Pathogenic microorganisms are believed to play a significant role in the impaired healing of chronic wounds leading to development of infection-related complications such as amputation, sepsis, and death (National Diabetes Fact Sheet, 2011). The amelioration of such severe conditions using potent indigenous medicinal alternatives from plants such as *Paullinia pinnata* can prove valuable in combating this menace and accompanying problems of antibiotic resistance and also, in alleviating the high cost of synthetic drugs which makes them less attractive options for management of such medical conditions. Thus, the antibacterial properties and mechanisms of action inherent in *Paullinia pinnata* Linn extract was elucidated in this study. The leaf extract obtained after ethanolic extraction was dark in color weighing approximately 108 g, representing about 8.31% of the powdered leaf sample (1300 g) used. Fractions obtained from column chromatography include n-hexane (5.4 g), ethyl acetate (4.5 g), acetone (1.7 g) and ethanol (0.9 g).

The antibacterial effect of crude ethanolic leaf extract of *P. pinnata* (Pp CE) and standard antibiotic (ampicillin) were used against test bacterial isolates. Out of the 24 test bacteria, 16 were susceptible to the Pp CE fractions at different concentrations tested as shown in Table 1. The zones of inhibition exhibited by the test isolates at concentration of 35 mg/mL ranged between 5.00 mm (*Macrococcus bruencis*) to 20.00 mm (*Staphylococcus muscae*). The pathogen *Enterobacter asburiae* was found resistant to ampicillin as opposed to other 20 susceptible isolates.

The antimicrobial effect of the fractions, both

singly and in combinations at 10 mg/mL shows that the multiple antibiotic-resistant *Enterobacter asburie, Klebsiella ozanae* and *Staphylococcus aureus* were susceptible to n-hexane fraction, ethyl acetate/n-hexane fraction of the plant extract. *Aerococcus suis* known to cause septicaemia meningitis was also susceptible to this plant leaf extract (Waters *et al.,* 2011). It was therefore, observed that the ethyl acetate fractions compared favourably with the standard antibiotic ampicillin, and thus, was selected for further extraction of *P. pinnata* bioactive components.

The n-hexane fraction at 10 mg/mL inhibited the growth of 15 out of the 16 susceptible wound isolates with the zones of inhibition ranging between 6.00 mm (*Citrobacter* sp.) and 22.00 mm (*Aerococcus suis*) but, *Streptococcus* sp. (C3) was resistant to this fraction. On the other hand, the ethyl acetate fraction inhibited the growth of 14 out of the 16 susceptible wound isolates while *S. canis and S. pyogenes* were resistant to this fraction. The zones of inhibition exhibited by the ethyl acetate fraction against susceptible test isolates ranged between 7.00 mm (*Macrococcus* sp.) and 28.00 mm (*Aerococcus suis*). The acetone and ethanol fractions were not active against any of these wound isolates.

For the synergetic antibacterial effect of ethyl acetate/n-hexane fractions *P. pinnata* leaf extract against the wound bacterial isolates, these combined active fractions recorded higher antibacterial activity of the plant extract on the wound isolates with a zone of inhibition range of 12.00 mm (*Macrococcus* sp) to 26.00 mm (*Aeococcus suis*).

The phytochemical analysis of crude leaf extract and ethyl acetate fraction of *P. pinnata* (Pp EA and Pp CE) revealed the presence of saponins, cardiac glycoside, resin, sterols, carbohydrates, phenols, alkaloids and terpenoids. Yusuf *et al.* (2013) has reported similar results for *P. pinnata* as a plant containing biologically active compounds that might be responsible for their chemo-preventive properties which include antioxidants, anticarcinogenic and anti-inflammatory effects (Akinyele *et al.,* 2011) thereby, contributing to the biocidal potentials of this plant extract against the wound isolates used for this study. The phytochemicals contained in the Pp CE fraction were the saponins, cardiac glycoside, carbohydrates, terpenoids, sterols, phenols, alkaloid and resin but tannin, flavonoids and phlobatannins were absent. The Pp EA fraction contained alkaloid, terpenoid, saponin, phenol, carbohydrate, and resin but not sterol and cardiac glycosides.

The presence of terpenoids has been reported in medicinal plants and found to promote cancer cell apoptosis indicating anti-cancer properties (Cox-Georgian *et al.*, 2019). *Paullinia pinnata* leaf extract tested positive for terpenoids which according to Onyeike *et al.* (2010), plays an important role in signaling and growth regulation of plants. In addition to this property, terpenoids has also displayed antibacterial properties through disruption of bacterial cell membranes (Dorman *et al.*, 2000). These phytochemicals are significantly involved in physiological responses in man (Edeago *et al.,* 2005).

Alkaloids in *P. pinnata* have displayed antibacterial activities on both *S. aureus* and *P. aeruginosa* (Donald *et al.,* 2015). Also, saponins in *P. pinnata* occur widely in many plant species (Jimoh *et al.*, 2007) and are considered key ingredients in traditional Chinese medicine responsible for majority of observed bioactive effects in medicinal plants (Liu and Henkel, 2002). They are usually stored in plant cells as inactive precursors but after pathogen attack, they are converted into biologically active antibiotics by plant enzymes which suggests that saponins in plants naturally offer protection against pathogen and pests (Singh *et al.*, 2007). Furthermore, another phytochemical compound extracted from *P. pinnata* leaf extract is sterol, which exhibit multiple and diverse physiological and pharmacological effects associated with the cardiovascular system (Singh and Kaushal, 2007).

Coupled with killing rate determination of the Pp EA fraction, other modes of action determined were nucleotide, potassium ion ($K^+$) and protein leakages. The fraction shown an appreciable leakage of nucleotide, potassium ion and protein from *Proteus vulgaris* and *Staphylococcus aureus'* cell membrane with increased cell contact time majorly attributed to antimicrobial compounds

present in *P. pinnata* leaf extract.

The results obtained showed that *P. pinnata* achieved a cidal effect on the wound isolates through disruption of their cell membrane. by Woo and Park, (2000) and Carson *et al.* (2002), reported that various compounds with antimicrobial properties damage the bacterial cytoplasmic membrane, thereby, causing loss of 260 nm absorbing materials as observed in the Pp EA fraction. The result obtained from this assay showed that the active fraction was able to leak more nucleotides of the two representative strains by damaging their cytoplasmic membranes at highest concentration of 2.86 µg/mL; 3 x MIC on *P. vulgaris* and, 2.33 µg/mL; 3 x MIC on *S. aureus* both at 120 min contact time.

Also, the Pp EA fraction caused potassium ions efflux from the isolates under study. The highest concentration of $K^+$ ions was leaked from *P. vulgaris* at 3 x MIC, 12.33 µg/mL while the concentration of 8.10 µg/mL at 3 x MIC leaked $K^+$ ions from *S. aureus* at 120 min contact time. The study by Grundling (2013), established that $K^+$ ions are essential for constant maintenance of internal pH and membrane potential of living cells, thus, leakage of these cations from bacterial cells after treatment with Pp EA fractions could have resulted from cytoplasmic membrane damage ultimately resulting in cell death.

The protein leakage from the cells of microorganisms caused by physical membrane rupture was assessed to determine the effect of Pp EA fraction on the representative strains in this study. The fraction leaked more proteins from *P. vulgaris* at highest concentration of 3 x MIC, 8.22 µg/mL and also, *S. aureus* at 3 x MIC, 6.91 µg/mL both at 120 min contact time. These observations can then suggest that the Pp EA fraction acts on the test cells (*S. aureus* and *P. vulgaris*) and caused their death through cytoplasmic disruption with increase in concentration and contact time, thus, indicating a monophasic effect as reported by Akinpelu *et al.* (2015).

Furthermore, the GC-MS analytical method was carried out to uncover the chemical compounds contained in the Pp EA active fraction, and about thirty compounds were detected among which are Trans-13-Octadecenoic acid (Khan *et al.*, 2022), Cholesta-4,6, dien-3-ol (Zhu *et al.*, 2018), a fat-soluble ascorbic acid; l-(+)-Ascorbic acid 2,6-dihexadecanoate (Shwaish *et al.*, 2019), Tetratriacontane (Ma *et al.*, 2018) and Beta-Sitosterol (Rashed, 2020); all capable of a wide range of antimicrobial activities. Trans-13-Octadecenoic acid was detected as the major component of the fractions at 23.25% abundance.

## CONCLUSION

The considerable antibacterial activities of both ethyl acetate and n-hexane fractions of *Paullinia pinnata* extract on wound isolates could be further explored especially, in combination to formulate new antimicrobial agents of natural origin. Also, potential drugs can be developed from this plant as potent interventions in treating wound infections and improving health care delivery.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest related to this article.

## AUTHORS' CONTRIBUTIONS

Olatujoye F., Oluduro A.O.: Conceptualization, Methodology, Software. Oluduro A.O.: Supervision. Olatujoye F.: Data curation, Resources, Writing- Original draft preparation. Bamigbade O.O., Oyedeji O., Idowu T.O.: Visualization, Investigation, Validation, Writing-Reviewing and Editing.

## REFERENCES


Akinpelu, D.A., Akinyele, T.A. and Okoh, A.I. (2011). In vitro anti-listerial properties crude aqueous and *n* hexane extract of the husk of Cocos nucifera. *African Journal of Biotechnology*, 10(41):8117-8121.

Akinpelu, D.A., Olayinka O.A., Akinpelu, O.F. and Okoh I.A. (2015a). Stem bark Extract and Fraction of *Persea americana* (Mill.) Exhibits Bactericidal Activities against Strains of *Bacillus cereus* associated with Food Poisoning. *Molecules,* 20:416-429.

Akinpelu, D.A., Alayande, K.A., Aiyegoro, O.A., Akinpelu, O.F., Okoh, A.I. (2015b). Probable mechanisms of biocidal action of *Cocos nucifera* Husk extract and fractions on bacteria Isolates. *BMC Compl Altern Med*, 15:116.

Burkill, H.M. (2000). The useful plants of West Tropical Africa. Royal Botanic Gardens.

Carson, C.F., Mee, B.J., and Riley, T.V. (2002). Mechanism of action of *Melaleuca alternifolia* (Tea Tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrobial Agents and Chemotherapy*, 46:1914–1920.

Clinical and Laboratory Standards Institute (CLSI), (2013). Performance standards for antimicrobial Susceptibility Testing. 22nd Informational Supplement 29:3. M100-522. Wayne, Pennsylvania.

Cox-Georgian, D., Ramadoss, N., Dona, C., Basu, C. (2019). Therapeutic and Medicinal Uses of Terpenes. Med. Plants Farm Pharm., 12:333–359.

Dorman, H.J.D., Deans, S.G. (2000). Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J. Appl. Microbiol.*, 88:308–316.

Edeago, H.O., Okwu, D.E. and Mbaebie, B.O. (2005). Phytochemical constituents of some Nigeria medicinal plants. African Journal of Biotechnology, 4:685-151.

Egbuna, C., Ifemeje, J.C., Udedi, S.C. and Kumar, S. (2019). Phytochemistry: Fundamentals, modern techniques and applications. Florida.

Evans, W.C. (2002). Trease and Evans Pharmacognosy, 15th edition. W.B Sauders Company Ltd, London. pp 137-139, 230-240.

Ghani, A. (1990). Introduction to Pharmacognosy. *pp* 1, 2, 187, 199-205.

Grundling, A. (2013). Potassium uptake systems in *Staphylococcus aureus:* new stories about ancient systems. *Microbiology,* 4(5):713-723.

Hawari, J., Halasz, A., Paquet, L., Zhou, E., Spencer, B., Ampleman, G., Thiboutot, S. (1998). Characterization of metabolites in the biotransformation of 2,4,6-trinitrotoluene with anaerobic sludge: role of triaminotoluene. *Appl Environ Microbiol.* 64(6):2200-2206.

Jimoh. F.O., Sofidiya, M.O., Afolayan, A.J. (2007). Antioxidant properties of the methanol extracts from the leaves of *Paullinia pinnata*. *J Med Food*, 10:707–711.

Khan, M.S., Uzair, M., Hanif, M., Jabeen, M., and Chaudhary, B.A. (2022). Anti-inflammatory potential of spectroscopically analyzed trans-13-octadecenoic acid of *Yucca elephantipes* Regel roots: in-vitro and in-vivo analysis. *Pakistan Journal of Pharmaceutical Sciences*, 35(6).

Lee, J.Y., Kim, Y.S. and Shin, D.H. (2002). Antimicrobial synergistic effect of linolenic acid and mono glyceride against *Bacillus cereus* and *Staphylococcus aureus*. *J. Agric. Food Chem.*, 50:2193–2199.

Lu, J. and Henkel, T. (2002). Traditional Chinese medicine (TCM): are polyphenols and saponins the key ingredients triggering biological activities? *Curriculum Medical Chemistry*, 9:1483-1485.

Ma, J., Xu, R.R., Lu, Y., Ren, D.F., and Lu, J. (2018). Composition, antimicrobial and antioxidant activity of supercritical fluid extract of Elsholtzia ciliata. Journal of Essential Oil-bearing Plants, 21(2):556–562.

Mabhiza, D., Chitemerere, T. and Mukanganyama, S. (2015). Antibacterial Properties of Alkaloid Extract from Callistemon citrinus and Vernonia adoensis against Staphylococcus aureus and Pseudomonas aeroginosa. *International Journal of Medicinal Chemistry*, 2016, pp. 7.

McMurry, C.V. (2000). Ph.D. Thesis. Biological and Phytochemical Investigation of Euphorbiaceae from Papua New Guinea. Swiss Federal Institute of Technology, Zurich. pp. 1-59

Mehta, M., Dutta, P. and Gupta, V. (2010). Bacterial isolates from burn wound infections and their antibiograms: an eight-year study. *Indian Journal Plastic Surgery,* 40:25-28.

Mulu, A., Moges F., Tessema, B. and Kassu, A. (2006). Pattern and multiple drug resistance of bacterial pathogens isolated from wound infection at University of Gondar Teaching Hospital, Northwest Ethiopia. *Ethiopia Medical Journal*, 44(2):125-131.

National Committee for Clinical Laboratory Standards, (2000). Methods for dilution, antimicrobial susceptibility tests for bacteria that grow aerobically, 5th edition volume 17. Approved standards-M7-A4. NCCLS document M7-A4. National Committee for Clinical Laboratory Standard Wayen Pa.

National Diabetes Fact Sheet, (2011). National estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.

Odenholt, I., Lowdinand, E. and Cars, O. (2001). Pharmacodynamics of telithromycin *in vitro* against respiratory tract pathogens. *Chemotherapy*, 45:23-29.

Odimegwu, D.C., Ibezim, E.C., Esimone, C.O., Nworu, C.S. and Okoye, F.B.C. (2008). Wound healing and antibacterial activities of the extract of *Dissotis theifoila* (*Melastomataceae*) stem formulated in a simple ointment base. *Journal Medicine Plant Review*, 2:11-16.

Oliphant, C.M. and Green, G.M. (2002). Quinolones: A comprehensive review. *American* Family Physician, 65:455-464.

Onyeike, E.N., Ikewuchi, J.C., Ikewuchi, C.C. and Uwakwe, A.A. (2010). Quantitative high-performance liquid chromatographic analysis of simple terpenes, carotenoids, phytosterols and flavonoids in the leaves of *Acalypha wilkesiena* muell arg. *Pakistan Journal of Science Technology*, 11(2):480-487.

Ousey, K. and McIntosh, C. (2009). Topical antimicrobial agents for the treatment of chronic wounds. *British J Community Nurs*; 14(9): S6, S8, S10.

Ozumba, U.C. (2007). Bacteriology of wound infections in the surgical wards of a teaching hospital in Enugu, Nigeria. *African Journal of Medical Science*, 36:341-344.

Rajan, K.P., Ranjan, N., Bansal, S.K. and Arora, D.R. (2011). Prevalence of *Pseudomonas* aeruginosa in post-operative wound infection. *Asian Pacific Journal of Tropical Biomedicine*, 370-375.

Rashed, K. (2020). Beta-sitosterol medicinal properties: A review article. *J. Sci. Innov. Technol*, 9, 208 - 212.

Shwaish, T., Al-Imarah, F.J., and Jasim, F.A. (2019). Wound healing capacity, antibacterial activity, and GC-MS analysis of *Bienertia sinuspersici* leaves extract. In Journal of Physics: Conference Series, 1294(6):062056. IOP Publishing.

Singh, K.N. and Kaushal, R. (2007). Comprehensive Notes on Commercial Utilization, characteristics and Status of Steroid Yielding Plants in India. *Ethnobotany Leaf*, 11:45-51.

Singh, B., Singh, J.P., Singh, N., Kaur, A. (2017). Saponins in pulses and their health promoting activities: a review. *Food Chemistry*, 233:540-549.

Thummel, K.E. and Shen, D.D. (2001). Design and optimization of dosage regimens: Pharmacokinetic data. In: Hardman JG, Limbird LE, Gilman AG, eds. Goodman & Gilman's. The pharmacological basis of theraupeutics. ed. New York: Mc Graw-Hill. 1924-2023.

Waters, A.E., Contente-Cuomo, T., Buchhagen, J., Liu, C.M., Watson, L., Pearce, K., Foster J.T., Bowers, J., Driebe, E.M., Engelthaler, D.M., Keim, P.S. and Price, L.B. (2011). Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clinical Infectious Dis*eases, 52(10):1227–1230.

Woo, I.S., Rhee, I.K., and Park, H.D. (2000). Differential damage in bacterial cells by microwave radiation on the basis of cell wall structure. *Applied and Environmental Microbiology*, 66:2243–2247

Yusuf, A.Z., Zakir, A., Shemau, Z., Abdullahi, M. and Halima, S.A. (2013). Phytochemical analysis of the methanol leaves extract of *Paullinia pinnata* Linn. *Journal of Pharmacognosy and Phytotherapy,* 6(2):10-16.

Zamble, A., Carpentier, M. and Kandoussi, A. (2006). *Paullinia pinnata* extracts rich in polyphenols promote vascular relaxation via endothelium-dependent mechanism. *Journal of Cardiovascular Pharmacology,* 47(4):599-608.

Zhu, Y.Z., Liu, J.W., Wang, X., Jeong, I.H., Ahn, Y.J., Zhang, C.J. (2018). Anti-BACE1 and antimicrobial activities of steroidal compounds isolated from marine *Urechis unicinctus*. *Marine drugs*, 16:94.