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# Determination of Antimicrobial, Antioxidant and Phytochemical Properties of *Cocos nucifera linn* Endocarp Extract on Bacteria Associated with Human Infection

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## Abstract

The study investigated that the antioxidant properties and mode of action of *Cocos nucifera* Linn endocarp extracts on pathogens associated with human infections with a view of producing natural product that serve as a potential template for new antibacterial against multiple resistant bacterial pathogens.

*Cocos nucifera* endocarp was collected from Ile-Ife, Osun State, Nigeria, and oven dried at 40°C for four days and ground into fine powder. The powdered sample was cold extracted using methanol and sterile distilled water in ratio 3:2 (v/v). The mixture obtained was concentrated *in vacuo* using rotary evaporator to drive out the organic solvent, while the aqueous layer was subsequently lyophilized. The crude extract obtained was screened for the antimicrobial activity against panel of bacterial strains implicated in human infections. The crude extract was later partitioned using four different organic solvents in order of their polarity. The antibacterial potentials of the crude extract along with the fractions obtained were determined using agar-well diffusion method. The minimum inhibitory concentration and minimum bactericidal concentration of the extracts against the bacterial strains were also determined. The rate of killing, protein, nucleotide and potassium ion leakage were determined using *Staphylococcus aureus* and *Escherichia coli* as representative of Gram positive and Gram negative bacteria respectively. The most active fraction which was ethyl acetate fraction was further partially purified by thin layer and column chromatography. The antimicrobial activity of the resulting samples was tested against the previously used bacterial strains. The most active sample of partially purified ethyl acetate fraction was analyzed using Gas Chromatography-Mass Spectrometry.

The results showed that the endocarp extract of *C. nucifera* and various fractions obtained from it exhibited varying degree of antibacterial activities. The phytochemical screening of the extract showed the presence of phenols, flavonoids, tannins, alkaloids, triterpenes and saponins. The minimum inhibitory concentration of the crude extract ranged between 0.27 and 8.75 mg/mL while the most active fraction ranged between 0.31 and 2.50 mg/mL. The rate of kill assay showed that the percentage of the cells killed increased with increasing concentration of the fraction and contact time intervals. Leakage of nucleotides, protein and potassium ions from test cells also followed the same trend observed for killing rate. *Cocos nucifera* endocarp extract exhibited 50% inhibition of free radicals at 0.011 mg/mL, whereas the ascorbic acid used as standard had $IC_{50}$ of 0.020 mg/mL. The major active constituent of the purified sample was identified as Ethyl Vanillin.

*Cocos nucifera* endocarp extracts which possessed antioxidant properties exhibited appreciable antibacterial activities against the test pathogens.

**Keywords:** Endocarp extract; Phytochemical screening; Antimicrobial activity; antioxidant; Pathogens

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## Introduction

The oldest written evidence of medicinal plant usage for preparation of drugs was found on a Sumerian clay slab from Nagpur which comprised 12 recipes for drug preparation referring to over 250 various plants [1]. Nearly 80% of people living in the developing countries especially in Africa depend on herbal medicine for their health needs including wounds, infectious and metabolic diseases [2]. Various plant parts, such as herbs, spices, fruit, vegetables and tropical plants have been showed to contain these natural antimicrobials which are of intense medical benefits [3]. Plant produces a wide variety of secondary metabolites which are natural bioactive compounds as a means of survival in a hostile environment [4] and are used as precursors or as the lead compounds in the pharmaceutical industry.

Africa is reputed for the extraordinary richness of its flora which constitutes thousands of species [5]. The research on the folklore uses of plants, cites that 75% may provide novel medicinal plants, which may lead to herbal drug discovery and formulation [6,7]. It is in record that the potential of plants in general and higher plants in particular as a source of new drugs has not been fully explored. The secondary metabolites (bio-active compounds) produced by these plants have been linked to their high medicinal potency and enable them to be used as a source of raw materials in the exploration of antimicrobial agents in the industry [8].

These antimicrobial compounds also serve as alternatives to formally approved chemically synthesized artificial drugs to which many infectious microorganisms have become resistant [9]. Some individual plant extract may have been subjected to specific pharmacological test (e.g. for cardiac activity only) however, the same extract may be examined for other types of activities such as pain relieving, anti-inflammation, antidiarrheal etc. [10].

The incidences of resistance of pathogens to existing antibiotics call for the search for new antimicrobials especially from natural origin. Accumulation of oxides in human system which ultimately leads to ageing could be controlled by the use of medicinal plants with promising source of antimicrobials and antioxidants. This study gave a quantitative evaluation on the antibacterial activities and mode of action of endocarp extract of *Cocos nucifera* found in the environment.

## Materials and Methods

### Materials

**Microorganisms:** The bacteria used in this study were obtained from culture collections of Prof. DA Akinpelu, Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The bacteria strains were:

**Gram positive**: *Bacillus cereus* (NCIB 6349), *Bacillus polymyxa* (LIO), *Bacillus stearothermophilus* (NCIB 8222), *Bacillus subtilis* (NCIB 6349), *Clostridium sporogenes* (NCIB 532), *Enterococcus faecalis*(NCIB 775), *Micrococcus luteus* (NCIB 196), *Shigelladysentariae* (LIO), *Staphylococcus aureus* (NCIB 8588) and *Streptococcus agalaticae* (CIRv).

**Gram negative**: *Escherichia coli* (NCIB 86), *Klebsiella pneumonia* (NCIB 418), *Pseudomonas aeruginosa* (NCIB 950), *Proteus mirabilis* (LIO), *Proteus vulgaris* (NCIB 67), *Pseudomonas fluorescens* (NCIB 3756), *Vibrio fluvialis* (LIO), *Vibrio furnissii* (LIO).

**Preparation of bacteria used for the experiment:** The bacterial strains used in the experiment were re-activated in nutrient broth and incubated at 37°C for 18 h. The organisms were stored on sterile nutrient agar slants in McCartney bottles at 4°C and subcultured at three months interval to maintain them for further use.

## Methods

**Drying and extraction of the plant sample:** The endocarp of *Cocos nucifera* used for this study was collected within Ile-Ife environment in Osun State, Nigeria in the month of July, 2016. The plant was identified in the Herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. The voucher number is IFE-1742. The endocarp was dried in hot air oven at 40°C until a constant weight of the sample was obtained. This was ground into fine powder. Exactly 2000 g of the powdered sample was extracted using methanol and sterile distilled water in ratio 3:2 (v/v) for four days with regular agitation. The supernatant collected was later filtered into a clean sterile dried conical flask. The filtrate was concentrated *in vacuo* to drive off the methanol and later lyophilized.

**Sensitivity testing of the crude extract and fractions of *C. nucifera* endocarp against bacterial strains:** The sensitivity testing of the extract was determined using agar-well diffusion method as described by Irobi et al. and Rusell and Furr with some modifications [11,12]. The bacterial strains were first grown in nutrient broth for 18 h before use. The 18 h old test organisms were then standardized using MacFarland ($10^8$ cfu/mL of 0.5 McFarland standards). The inoculum was then streaked onto an already sterilized Mueller-Hinton agar (Lab M) plate. Wells were then bored into the medium using a sterile 6 mm cork borer. The wells were filled up with 35 mg/mL prepared solution of the extract. Care was taken not to allow solution of the extract to spill on the surface of the medium. The plates were then allowed to stand on the laboratory bench for about 1-2 h to allow proper inflow of the solution into the medium before incubating the plate in an incubator at 37°C for 24 h. The plates were later observed for the zones of inhibition. The effects of the extracts on bacterial strains were compared with standard antibiotic; streptomycin and ampicillin.

**Determination of minimum inhibitory concentrations (MICs) of the crude extract and the fractions of *C. nucifera* endocarp on bacterial strains:** The MICs of the extract was determined using the method described by Akinpelu and Kolawole [13]. Two-fold dilution of the extract was prepared and 2 mL of different concentrations of the solution was added to 18 mL of pre-sterilized molten nutrient agar to give final concentration range of 0.273 to 35.0 mg/mL. The medium was then poured into sterile petri dishes and allowed to set. The surfaces of the media were allowed to dry before streaking with 18 h old standardized bacterial cultures. The plates were later incubated at 37°C for up

to 72 h after and were examined for the presence or absence of growth. Minimum inhibitory concentration was taken as the lowest concentration that prevented bacterial growth.

**Determination of minimum bactericidal concentrations (MBCs) of the crude extract of *C. nucifera* and the fractions on bacterial strains:** Minimum bactericidal concentrations of the extract were determined in accordance with the method of Olorundare et al. with some modifications [14]. 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 48 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 48 h incubation period.

**Phytochemical screening of the extract:** Endocarp crude extract of the plant was subjected to phytochemical screening using Trease, Evans and Haborne, Baxter to test for alkaloids, tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycoside [15,16]. Viable counts of the test organisms were initially determined. A 0.5 mL volume of known cell density (by viable counts of $10^8$ cfu/mL) from each organism suspension was added to 4.5 mL of different concentration of the fractions. The suspension was thoroughly mixed and held at room temperature (28-30°C) and the killing rate was determined over a period of 2 h. Exactly 0.5 mL volume of each suspension was withdrawn at a time intervals and transferred to 4.5 mL of nutrient broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compounds carry-over from the test suspensions. The suspension was properly shaken then serially diluted in sterile physiological saline. Exactly 0.5 mL of the final dilution of the test organism was transferred into pre-sterilized nutrient agar at 45°C and plated out. The plates were allowed to set and incubated upside down at 37°C for 72 h. Control experiment was set up without inclusion of antimicrobial agent. Viable counts were made in triplicates of each sample.

**Determination of potassium ions leakage from the test bacterial strains using active fractions:** The method of Allwood, Hugo and Gale was used for this assay [17,18]. Cells of *E. coli* and *S. aureus* from 18 hr old nutrient broth culture were washed in 0.09 w/v NaCl (normal saline). Washed suspension of *E. coli* and *S. aureus* (approximately $10^8$ cells) were treated with various concentrations of the fractions relative to MIC at various time intervals for 2 h. Each suspension was then centrifuged at 10,000 rpm 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.

**Determination of nucleotides leakage from the test strains by the active fractions:** The method described by Joswick et al. with some modification was used to determine the leakage of the nucleotides from the test cells. Cells of *E. coli* and *S. aureus* from 18 hr old nutrient broth culture were washed in 0.9% w/v normal saline [19]. Washed suspension of *E. coli* and *S. aureus* (inoculums approximately $10^8$ cells) were treated with various concentrations of the fractions relative to MICs at various time intervals for 2 h. Each suspension was then centrifuged at 10,000 rpm and the optical density of the supernatant collected was measured at 260 nm wavelength using spectrophotometer. Normal saline inoculated with the same quantity of inoculum was used as control.

**Determination of protein leakage from the test strain by the active fraction:** Cells of *E. coli* and *S. aureus* from 18 hr nutrient broth culture were separately washed in 0.9% w/v normal saline. Washed suspension of *E. coli* and *S. aureus* (inoculums size approximately $10^8$ cells 0.5 Mcfarland standards) were treated with various concentration of the fraction relative to MICs at various time interval of 2 h. Each suspension was then centrifuged at 7000 rpm and supernatant collected was assayed for protein using Bradford method [20]. In assaying for protein, 0.4 mL Bradford reagent was added to 1.6 mL sample (0.2 mL supernatant plus 1.4 mL sterile distilled water) to make up 2 mL total volume. Optical density (OD) of the resulting solution was thereafter taking at 595 nm after 5 min. The optical density of each of the samples was calculated from the equation of the best linear regression line obtained from the graph of Bovine Serum Albumin (BSA) standard curve.

**Preparation of Bovine serum albumin standard curve:** Bovine serum albumin stock solution of concentration 100 µg/mL was first prepared. Varying concentration of the bovine serum albumin was thus prepared from the stock solution. Bradford reagent (0.4 mL) was added to the various bovine serum albumin concentrations. This was allowed to stand for five minute after which the optical density was measured at 600 nm. The various optical density obtained were thereafter plotted against bovine serum albumin concentrations to form standard albumin curve. The concentrations of protein in the samples were then calculated from the equation of best-fit linear regression line obtained from the graph of the bovine serum albumin standard curve.

**DPPH antioxidant assay of *C. nucifera*:** The antioxidant activity (AA%) of each substance was assessed by DPPH free radical assay. The measurement was performed as described by Brand-williams et al. [21]. Endocarp extract of *C. nucifera* was reacted with DPPH radical in ethanol solution. The 0.5 mL of the extract was mixed with 3 mL of absolute ethanol and 0.3 M DPPH radical solution of 0.5 mM solution in ethanol. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The colour change when DPPH reacted with the extract was read at 517 nm after 30 min of reaction using UV spectrophotometer. The mixture of ethanol (3.3 mL) and sample (0.5 mL) served as blank. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL). The scavenging activity percentage (AA%) was determined according to Mensor et al. [22].

$$\text{DPPH radical scavenging activity \%} = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100$$

**Partial purification of the fractions using thin layer and column chromatography:** The best solvent system for the elution of ethyl acetate fraction on column chromatography was determined by eluting the fractions with different solvents systems on thin layer chromatography (TLC) plate. To do this, solvent was poured into developing chamber to 0.5 cm depth. Part of the inside of the

chamber was lined with filter paper, covered with lid, swirl gently and allowed to stand till TLC plate was prepared. The TLC plate was cut out into a convenient size without disturbing the coating on the adsorbent. A line which served as the origin was carefully at 0.5 cm mark above the bottom of the plate with pencil. The ethyl acetate fraction (1 mg) was dissolved in 1 mL of methanol and the resulting solution was carefully spotted on the prepared TLC plate at the origin with micro-capillary tube. The spotted plate was afterwards developed in TLC chamber. The TLC plate was allowed to develop in the chamber until solvent front was about 0.5 cm below the top of the TLC plate. The TLC plate was removed; solvent front marked carefully with pencil and allowed to dry. The plate was visualized under UV-light at 254 and 366 nm to view any fluorescence. The spots observed were circled with pencil. The solvent system determined for ethyl acetate fraction elution base on the $R_f$ values was in order of n-hexane, n-hexane-ethyl acetate (1:1), n-hexane-ethyl acetate (2:8), n-hexane-ethyl acetate (1:9), ethyl acetate, ethyl acetate-methanol (9:1), ethyl acetate-methanol (8:2) and ethyl acetate-methanol (7:3).

Thereafter, exactly 5 g of the ethyl acetate fraction was dissolved in a minimal amount of methanol adsorbed on 25 g of silica gel of 60-200 mesh size and then allowed to dry. It was then chromatographed on silica gel column (650 x 40 mm) and gradiently eluted with pre-determined solvent system. Column chromatography of ethyl acetate fraction yielded ETHYL-A, ETHYL-B, ETHYL-C and ETHYL-D partially purified samples. The elutes were collected in test tubes, analysed using TLC to determine fractions with similar retention factors ($R_f$). Fractions with similar retention factors were bulked together, concentrated to dryness *in vacuo* and stored in a container in refrigerator for further use. Antimicrobial activity of the partially purified fractions was carried out using the method as described previously.

**Gas chromatography-mass spectrometry analysis of partially purified ethyl acetate fraction of *C. nucifera* endocarp extract:** Gas chromatography-Mass spectrometry (GC-MS) analysis of the partially purified ethyl acetate fraction of *C. nucifera* was performed using Agilent technologies GC system comprising on AOC-20i auto-sampler and a Gas chromatograph interfaced to a triple axis mass spectrometer detector equipped with an Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused to a capillary column (30 x 0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) 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˚C, the ion-source temperature was 200˚C, the oven temperature was programmed from 110˚C (isothermal for 2 min) with an increase of 10˚C/min to 200˚C, then 5˚C/min to 280˚C, ending with a 9 min isothermal at 280˚C. Mass spectra were taken at70 eV; a scan interval of 0.5 second. The solvent delay was 0 to 6 min, and the total GC/MS running time was 34.667 seconds. The relative percentage amount of each component was calculated by comparing its average peak to the total areas. 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.

## Results

The extract collected was dark brown in colour and the yield was 110.50 g (5.53%).

### Antibacterial activities of crude extract of *c. nucifera* endocarp against various bacterial strains

Sensitivity patterns exhibited by the crude extract of *C. nucifera* against bacterial strains was used for this study as shown in **Table 1**. Nineteen out of the twenty-five bacterial strains tested were susceptible to the activity of the extract at a final concentration of 35 mg/mL. The test bacteria strains that were resistant to the extract included *Enterococcus faecalis*, *Escherichia coli* (CIU), *Staphylococcus aureus* (CIU), *Pseudomonas aeruginosa* (CIU), *Salmonella typhi* (LIO), *Vibrio fluvialis* (LIO). The zones of inhibition exhibited by the extract against the test organisms ranged between 8.67 mm and 18.00 mm. The highest zone of inhibition (18.00 mm) was exhibited against *Micrococcus luteus* (NCIB 196) while the lowest zone of inhibition (8.67 mm) was exhibited against *P. fluorescens*. On the other hand, all organisms tested against the standard antibiotics-streptomycin and ampicillin at a concentration of 1 mg/mL were susceptible to these compounds except *Clostridium sporogenes*, *E. coli* (CIU), *Plesiomonas shigelloides* (LIO), *P. aeruginosa* (CIU), *P. Fluorescens* (NCIB 3756), *S. aureus* (CIU) and *Streptococcus agalatiae* (CIRv) that was resistant to streptomycin. *Klebsiella pneumoniae* (NCIB 418), *M. luteus* (NCIB 196), *P. aeruginosa* (NCIB 950), *P. aeruginosa* (CIU), *S. aureus* (CIU), *V. fluvialis* and *V. furnissii* (LIO) were resistant to ampicillin.

### Antimicrobial activities exhibited by fractions obtained from crude extract of *C. nucifera* on the test bacterial strains

Three fractions obtained from the crude extract of *C. nucifera* endocarpand these are ethyl acetate, butanol and aqueous fractions. Chloroform and n- hexane did not show affinity for the bioactive components of the extract. The antimicrobial activity of various fractions from *C. nucifera* crude extract is shown in **Table 2**.

The aqueous, butanol and ethyl acetate fractions exhibited antimicrobial activities against various bacterial strains used for this study. The zones of inhibition exhibited by these fractions against the test organisms ranged between 8.33 mm and 20.33 mm. The Ethyl acetate fraction showed the highest antimicrobial activity among these fractions. This fraction inhibited the growth of nineteen out of the twenty-five test organisms. The zone of inhibition exhibited by ethyl acetate fraction against various *Bacillus* species ranged between 10.66 mm and 17.66 mm. On the other hand, zones of inhibition observed for strains of *E. coli*

**Table 1:** The sensitivity patterns exhibited by crude extract of *C. nucifera* and the standard antibiotics against the test strains.

| Zone of inhibition (mm)** | | | | |
|---|---|---|---|---|
| Bacterial Strains | Crude extract (35 mg/mL) (Mean ± SD) | Streptomycin (1 mg/mL) (Mean ± SD) | Ampicillin (1 mg/mL) (Mean ± SD) | M/W 01:01 |
| *Bacillus cereus* (NCIB 6349) | 15.00 ± 1.63 | 28.00 ± 1.73 | 20.70 ± 1.10 | 0 |
| *Bacillus polymyxa* (LIO) | 12.00 ± 0.00 | 17.33 ± 1.73 | 0 | 0 |
| *Bacillus stearothermophilus*(NCIB 8222) | 14.33 ± 0.94 | 27.67 ± 2.08 | 16.33 ± 1.53 | 0 |
| *Bacillus subtilis* (NCIB 3610) | 13.33 ± 1.54 | 28.33 ± 1.53 | 16.00 ± 3.61 | 0 |
| *Clostridium sporogenes* (NCIB 532) | 10.66 ± 0.94 | 0 | 14.00 ± 2.51 | 0 |
| *Corynebacterium pyogenes* (LIO) | 10.66 ± 0.94 | 20.33 ± 2.31 | 21.00 ± 2.65 | 0 |
| *Enterococcus faecalis* (NCIB 775) | 0 | 20.67 ± 2.52 | 17.33 ± 2.52 | 0 |
| *Escherichia coli* (NCIB 86) | 14.00 ± 0.94 | 20.33 ± 2.31 | 20.67 ± 1.15 | 0 |
| *Escherichia coli* (CIU) | 0 | 0 | 18.70 ± 1.20 | 0 |
| *Escherichia coli* (CISt) | 10.66 ± 0.94 | 21.00 ± 2.65 | 0 | 0 |
| *Klebsiella pneumoniae* (NCIB 418) | 15.33 ± 1.88 | 20.67 ± 2.52 | 0 | 0 |
| *Micrococcus luteus* (NCIB 196) | 18.00 ± 0.94 | 22.33 ± 2.52 | 16.33 ± 2.89 | 0 |
| *Plesiomonas shigelloides* (LIO) | 15.00 ± 1.41 | 0 | 0 | 0 |
| *Proteus mirabilis* (CIU) | 12.00 ± 0.00 | 19.00 ± 2.65 | 17.67±2.52 | 0 |
| *Proteus vulgaris* (CIU) | 10.00 ± 0.67 | 16.67 ± 2.08 | 16.33 ± 3.21 | 0 |
| *Pseudomonas aeruginosa* (NCIB 950) | 10.00 ± 0.00 | 18.00 ± 2.00 | 0 | 0 |
| *Pseudomonas aeruginosa* (CIU) | 0 | 0 | 0 | 0 |
| *Pseudomonas fluorescens* (NCIB3756) | 8.67 ± 0.94 | 0 | 20.70 ± 1.10 | 0 |
| *Salmonella typhi* (LIO) | 0 | 20.33 ± 2.31 | 19.00 ± 2.65 | 0 |
| *Shigella dysentariae* (LIO) | 16.66 ± 0.94 | 25.30 ± 1.10 | 24.70 ± 1.30 | 0 |
| *Staphylococcus aureus* (NCIB 8588) | 16.00 ± 1.88 | 22.33 ± 2.52 | 27.30 ± 1.20 | 0 |
| *Staphylococcus aureus* (CIU) | 0 | 0 | 0 | 0 |
| *Streptococcus agalaticae* (CIRv) | 10.00 ± 1.88 | 0 | 24.70 ± 1.30 | 0 |
| *Vibrio fluvialis* (LIO) | 0 | 17.30 ± 1.20 | 0 | 0 |
| *Vibrio furnissii* (LIO) | 10.00 ± 1.88 | 26.70 ± 2.30 | 0 | 0 |

**Abbreviations:** CIRv: Clinical Isolates from Rectovagina, CISt: Clinical Isolates from Stool, CIU: Clinical Isolates from Urine, NCIB: National Collection of Industrial Bacteria, LIO: Locally Isolated Organism, mm**: Mean of three replicates, M/W: Methanol/Water, 0: Not sensitive.

ranged between 9.66 and 18.00 mm. *Plesiomonas shigelloides*, *Pseudomonas aeruginosa* (CIU), *Salmonella typhi*, *Shigella dysentariae* and *Vibrio furnissii* were not susceptible to the ethyl acetate fraction. On the other hand, butanol and aqueous fractions showed antibacterial activities against six and three test organisms respectively.

## Susceptibility patterns exhibited by partially purified ethyl acetate fractions against the test bacterial strains

Susceptibility patterns exhibited by partially purified fraction of ethyl acetate against the organisms are shown in **Table 3**. The partially purified Ethyl-D was not active against the bacterial strains while Ethyl A, Ethyl B and Ethyl C showed varying degrees of activities. Ethyl C showed the highest antimicrobial activity among these partially purified samples.

Ethyl- A exhibited antibacterial activity against one out of the twenty-five test bacterial strains used for this study while Ethyl-B exhibited activity against five of the twenty-five bacterial strains and the zones of inhibition against the bacterial strains ranged between 8.66 mm and 18.00 mm. The Ethyl-C exhibited activity against nine out of the twenty-five test bacterial strains and the zones of inhibitions ranged between 9.67 mm and 17.33 mm. The susceptible bacterial strains are shown in **Table 3**. Other partially purified sample, that is, Ethyl-D did not show activity on any of the test organisms.

## The minimum inhibitory concentrations and the minimum bactericidal concentrations exhibited by crude extract of *C. nucifera* against susceptible bacterial strains

The minimum inhibitory concentrations and the minimum bactericidal concentrations exhibited by the crude extract of *C. nucifera* against susceptible bacterial strains are shown in **Table 4**. The MICs of the crude extract against the susceptible bacterial strains ranged between 0.27 mg/mL and 8.75mg/mL while the lowest MICs of 0.27 mg/mL were observed against *B. cereus* (NCIB 6349) and *E. coli* (NCIB 86). The highest MIC of 8.75 mg/mL was observed against *P. aeruginosa* (NCIB 950). The MBCs of the crude extract against the susceptible bacterial strains ranged between 1.09 mg/mL and 17.5 mg/mL while the lowest MBCs of 1.09 mg/mL were observed against *B. cereus* (NCIB 6349) and *S. Aureus* (NCIB 8588). The highest MBC of 17.50 mg/mL was observed against *P. aeruginosa* (NCIB 950).

**Table 2:** The sensitivity patterns exhibited by fractions collected from *C. nucifera* crude extract on the test bacterial strains.

| Zone of inhibition (mm)** | | | | |
|---|---|---|---|---|
| Bacterial Strains | Aqueous (10 mg/mL) (Mean ± SD) | Butanol (10 mg/mL) (Mean ± SD) | Ethyl acetate (10 mg/mL) (Mean ± SD) | M/W 01:01 |
| *Bacillus cereus* (NCIB6349) | 0 | 13.33 ± 0.94 | 17.66 ± 1.89 | 0 |
| *Bacillus polymyxa* (LIO) | 0 | 14.00 ± 0.94 | 15.00 ± 2.37 | 0 |
| *Bacillus stearothermophlus* (NCI 822) | 0 | 20.33 ± 1.41 | 10.66 ± 0.94 | 0 |
| *Bacillus subtilis* (NCIB 3610) | 0 | 0 | 13.00 ± 2.00 | 0 |
| *Clostridium sporogenes* (NCIB 532) | 0 | 0 | 0 | 0 |
| *Corynebacterium pyogenes* (LIO) | 0 | 0 | 15.00 ± 2.35 | 0 |
| *Enterococcus faecalis* (NCIB 775) | 0 | 0 | 14.33 ± 3.77 | 0 |
| *Escherichia coli* (NCIB 86) | 14.00 ± 0.94 | 10 | 18 | 0 |
| *Escherichia coli* (CIU) | 0 | 0 | 15.00 ± 0.47 | 0 |
| *Escherichia coli* (CISt) | 0 | 0 | 9.66 ± 0.47 | 0 |
| *Klebsiella pneumoniae* (NCIB 418) | 8.33 ± 0.47 | 18.00 ± 0.94 | 10.67 ± 0.58 | 0 |
| *Micrococcus luteus* (NCIB 196) | 0 | 0 | 14.67 ± 1.53 | 0 |
| *Plesiomonas shigelloides* (LIO) | 0 | 0 | 0 | 0 |
| *Proteus mirabilis* (CIU) | 0 | 0 | 11.00 ± 2.00 | 0 |
| *Proteus vulgaris* (CIU) | 0 | 0 | 17.33 ± 2.82 | 0 |
| *Pseudomonas aeruginosa* (NCIB 950) | 0 | 0 | 13.67 ± 1.15 | 0 |
| *Pseudomonas aeruginosa* (CIU) | 0 | 0 | 0 | 0 |
| *Pseudomonas fluorescens* (NCIB356) | 0 | 0 | 12.66 ± 3.77 | 0 |
| *Salmonella typhi* (LIO) | 0 | 0 | 0 | 0 |
| *Shigella dysentariae* (LIO) | 0 | 0 | 0 | 0 |
| *Staphylococcus aureus* (NCIB 8588) | 13.66 ± 1.41 | 10.66 ± 0.94 | 14.67 ± 1.53 | 0 |
| *Staphylococcus aureus* (CIU) | 0 | 0 | 17.33 ± 2.08 | 0 |
| *Streptococcus agalaticae* (CIRv) | 0 | 0 | 14.67 ± 1.53 | 0 |
| *Vibrio fluvialis* (LIO) | 0 | 0 | 9.67 ± 1.15 | 0 |
| *Vibrio furnissii* (LIO) | 0 | 0 | 0 | 0 |

**Abbreviations:** CIRv: bClinical Isolates from Rectovagina, CISt: Clinical Isolates from Stool, CIU: Clinical Isolates from Urine, NCIB: National Collection of Industrial Bacteria, LIO: Locally Isolated Organism, mm**:** Mean of three replicates, M/W: Methanol/Water, 0: Not sensitive.

## The minimum inhibitory and the minimum bactericidal concentrations exhibited by ethyl acetate fractions against the susceptible bacterial strains

The ethyl acetate fraction exhibited appreciable antimicrobial activity when compared to those shown by aqueous and butanol fractions. Thus ethyl acetate fraction was chosen for further tests. Various MIC and MBCs were exhibited by ethyl acetate fraction against the susceptible test organisms (**Table 5**). The MICs and MBCs were exhibited by this fraction against the organisms ranged between 0.31 mg/mL and 2.50 mg/mL. The lowest MIC of 0.31 mg/mL was observed against *E. coli* (CISt) and the highest MICs of 2.50 mg/mL were observed against *B. cereus* (NCIB 6349), *B. polymyxa* (LIO) and *Micrococcus luteus* (NCIB 196). The Minimum bactericidal concentrations (MBC) exhibited by this fraction ranged between 0.63 mg/mL and 5.00 mg/mL.

## Phytochemical screening of the plant extract

Phytochemical screening of the extract showed the presence of saponins, tannins, flavonoids, alkaloids, triterpenes and phenols as shown in **Table 6**.

## The extent and rate of killing of *S. aureus* exhibited by ethyl acetate fraction

The extent and rate of killing of *S. aureus* by ethyl acetate fraction at 1 x MIC, 2 x MIC and 3 x MIC concentrations indicated in **Figure 1**. Its percentage of the organism killed by the fraction at 1 x MIC concentration in 15 min was 30.1% while the percentage of cell killed at 30 min rose to 50%. After 60 min of contact time with this fraction, the percentage of the organisms killed was 57.9%. As the contact time increased to 90 min, the percentage of the cells killed was 70.6% and this rose to 86.8% after 120 min of contact time. When the concentration of the ethyl acetate fraction was doubled, the percentage of organisms killed was 41.7% at 15 min of the contact time. At 30 min of contact time, the percentage of the cells killed increased to 73.3%; and with an increase in the contact time to 60 min, the percentage of organisms killed was 85%.

Each point represents the mean $\log_{10}$ survival of bacterial cells at a particular time interval in the presence of the fraction. After 90 min of the contact time interval, the percentage of the organisms killed has increased to 90.2%, while it rose to 95.5% after 120 min of contact time. The extent and rate of kill of the

**Table 3:** Antimicrobial activities of partially purified fractions of *C. nucifera* extract against bacterial strains.

| Zone of inhibition (mm)** | | | | |
|---|---|---|---|---|
| Bacterial Strains | Aqueous (10 mg/mL) (Mean ± SD) | Butanol (10 mg/mL) (Mean ± SD) | Ethyl acetate (10 mg/mL) (Mean ± SD) | M/W 01:01 |
| *Bacillus cereus* (NCIB6349) | 0 | 13.33 ± 0.94 | 17.66 ± 1.89 | 0 |
| *Bacillus polymyxa* (LIO) | 0 | 14.00 ± 0.94 | 15.00 ± 2.37 | 0 |
| *Bacillus stearothermophlus* (NCI 822) | 0 | 20.33 ± 1.41 | 10.66 ± 0.94 | 0 |
| *Bacillus subtilis* (NCIB 3610) | 0 | 0 | 13.00 ± 2.00 | 0 |
| *Clostridium sporogenes* (NCIB 532) | 0 | 0 | 0 | 0 |
| *Corynebacterium pyogenes* (LIO) | 0 | 0 | 15.00 ± 2.35 | 0 |
| *Enterococcus faecalis* (NCIB 775) | 0 | 0 | 14.33 ± 3.77 | 0 |
| *Escherichia coli* (NCIB 86) | 14.00 ± 0.94 | 10 | 18 | 0 |
| *Escherichia coli* (CIU) | 0 | 0 | 15.00 ± 0.47 | 0 |
| *Escherichia coli* (CISt) | 0 | 0 | 9.66 ± 0.47 | 0 |
| *Klebsiella pneumoniae* (NCIB 418) | 8.33 ± 0.47 | 18.00 ± 0.94 | 10.67 ± 0.58 | 0 |
| *Micrococcus luteus* (NCIB 196) | 0 | 0 | 14.67 ± 1.53 | 0 |
| *Plesiomonas shigelloides* (LIO) | 0 | 0 | 0 | 0 |
| *Proteus mirabilis* (CIU) | 0 | 0 | 11.00 ± 2.00 | 0 |
| *Proteus vulgaris* (CIU) | 0 | 0 | 17.33 ± 2.82 | 0 |
| *Pseudomonas aeruginosa* (NCIB 950) | 0 | 0 | 13.67 ± 1.15 | 0 |
| *Pseudomonas aeruginosa* (CIU) | 0 | 0 | 0 | 0 |
| *Pseudomonas fluorescens* (NCIB356) | 0 | 0 | 12.66 ± 3.77 | 0 |
| *Salmonella typhi* (LIO) | 0 | 0 | 0 | 0 |
| *Shigella dysentariae* (LIO) | 0 | 0 | 0 | 0 |
| *Staphylococcus aureus* (NCIB 8588) | 13.66 ± 1.41 | 10.66 ± 0.94 | 14.67 ± 1.53 | 0 |
| *Staphylococcus aureus* (CIU) | 0 | 0 | 17.33 ± 2.08 | 0 |
| *Streptococcus agalaticae* (CIRv) | 0 | 0 | 14.67 ± 1.53 | 0 |
| *Vibrio fluvialis* (LIO) | 0 | 0 | 9.67 ± 1.15 | 0 |
| *Vibrio furnissii* (LIO) | 0 | 0 | 0 | 0 |

**Abbreviations:** CIRv: Clinical Isolates from Rectovagina, CISt: Clinical Isolates from Stool, CIU: Clinical Isolates from Urine, NCIB: National Collection of Industrial Bacteria, LIO: Locally Isolated Organism, mm**:** Mean of three replicates, M/W: Methanol/Water, 0: Not sensitive.

**Table 4:** The minimum inhibitory concentrations and minimum bactericidal concentrations of the crude extract exhibited against the test bacterial strain.

| Bacterial Strains | Crude extract | |
|---|---|---|
| | MIC (mg/mL) | MBC (mg/mL) |
| *Bacillus cereus* (NCIB 6349) | 0.27 | 1.09 |
| *Bacillus polymyxa* (LIO) | 2.19 | 8.75 |
| *Bacillus sterothermophilus* (NCIB 822) | 2.19 | 8.75 |
| *Clostridium sporogenes* (NCIB 532) | 4.38 | 8.75 |
| *Corynebacterium pyogenes* (LIO) | 4.38 | 8.75 |
| *Escherichia coli* (NCIB 86) | 0.27 | 4.38 |
| *Escherichia coli* (CISt) | 0.54 | 2.19 |
| *Klebsiella pneumoniae* (NCIB 418) | 1.09 | 2.19 |
| *Micrococcus luteus* (NCIB 196) | 4.38 | 8.75 |
| *Plesiomonas shigelloides* (LIO) | 2.19 | 8.75 |
| *Proteus mirabilis* (LIO) | 2.19 | 4.38 |
| *Proteus vulgaris* (LIO) | 2.19 | 4.38 |
| *Pseudomonas aeruginosa*(NCIB950) | 8.75 | 17.5 |
| *Pseudomonas fluorescens*(NCIB3756 | 2.19 | 8.75 |
| *Shigella dysentariae* (LIO) | 1.09 | 2.19 |
| *Staphylococcus aureus* (NCIB8588) | 0.55 | 1.09 |
| *Streptococcus agalaticae* (CIRV) | 4.38 | 8.75 |
| *Vibrio furnissii* (LIO) | 4.38 | 8.75 |

**Abbreviations:** CIRv: Clinical Isolates from Rectovagina, CISt: Clinical Isolates from Stool, CIU: Clinical Isolates from Urine, NCIB: National Collection of Industrial Bacteria, LIO: Locally Isolated Organism.

fraction at 3 x MIC concentration followed the same trend with the concentrations aforementioned. As the concentrations of the fraction increased with increase in time, the percentage of the organisms killed also increased.

## The extent and rate of killing of *E. coli* exhibited by the ethyl acetate fraction

The extent and rate of killing of *E. coli* by ethyl acetate fraction at 1 x MIC, 2 x MIC and 3 x MIC concentration indicated in **Figure 2**. The percentage of the organisms killed by the fraction at 1 x MIC concentration in 15 min was 5.1%, the percentage of the cells killed at 30 min rose to 24.1%. After 60 min of contact time with this fraction, the percentage of the cells killed was 47.6%. When the contact time was increased to 90 min, the percentage of the cells killed was 65.1% and this rose to 82.5% after 120 min of contact time. When the concentration of the fraction was doubled, the percentage of organisms killed by the fraction within 15 min was 15.1%. At 30 min of contact time, the percentage of cells killed increased to 49.1%; and with the increase in the contact time to 60 min, the percentage of organism killed got to 67.9%. After 90 min of the contact time interval, the percentage of the organisms killed has increased to 89.6% while it rose to 94.8% after 120 min of contact time. The extent and rate of killing by the fraction at 3 x MIC concentration followed the same trend as in previous test.

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

**Table 5:** The minimum inhibitory concentrations and minimum bactericidal concentrations of the ethyl acetate fraction against the test bacterial strains.

| Bacterial Strains | Ethylacetate fraction | |
|---|---|---|
| | MIC (mg/mL) | MBC (mg/mL) |
| *Bacillus cereus* (NCIB 6349) | 2.50 | 5.00 |
| *Bacillus polymyxa* (LIO) | 2.50 | 5.00 |
| *Bacillus stearothermophilus* | 1.25 | 5.00 |
| *Bacillus subtilis* (NCIB 3610) | 1.25 | 5.00 |
| *Corynebacterium pyogenes* (LIO) | 0.63 | 1.25 |
| *Enterococcus faecalis* (NCIB 775) | 0.63 | 1.25 |
| *Escherichia coli* (NCIB 86) | 1.25 | 2.50 |
| *Escherichia coli* (CIU) | 1.25 | 2.50 |
| *Escherichia coli* (CISt) | 0.31 | 0.63 |
| *Klebsiella pneumoniae* (NCIB 418) | 1.25 | 2.50 |
| *Micrococcus luteus* (NCIB 196) | 2.50 | 5.00 |
| *Plesiomonas shigelloides* (LIO) | 2.50 | 5.00 |
| *Proteus mirabilis* (CIU) | 2.50 | 5.00 |
| *Proteus vulgaris* (CIU) | 2.50 | 5.00 |
| *Pseudomonas fluorescens* (NCIB3756) | 1.25 | 2.50 |
| *Staphylococcus aureus* (NCIB 8588) | 0.63 | 1.25 |
| *Staphylococcus aureus* (CIU) | 0.63 | 1.25 |
| *Streptococcus agalaticae* (CIRv) | 1.25 | 2.50 |
| *Vibrio fluvialis* (LIO) | 2.50 | 5.00 |

**Abbreviations:** CIRv: Clinical Isolates from Rectovagina, CISt: Clinical Isolates from Stool, CIU: Clinical Isolates from Urine, NCIB: National Collection of Industrial Bacteria, LIO: Locally Isolated Organism.

**Table 6:** Phytochemical compounds revealed from *Cocos nucifera* extract.

| Chemical Test | Result |
|---|---|
| Alkaloids | Positive |
| Cardiac glycosides | Negative |
| Flavonoids | Positive |
| Phenols | Positive |
| Triterpenes | Positive |
| Steroids | Negative |
| Saponins | Positive |
| Tannins | Positive |

## The effect of ethyl acetate fraction on potassium leakage from *S. aureus* Cells

The potassium ion leakages from *S. aureus* cells due to the effect of ethyl acetate fraction at 1 x MIC, 2 x MIC, 3 x MIC concentrations indicated in **Figure 3**, where amount of potassium ion leaked from *S. aureus* cells at 1 x MIC in 15 min was 0.91 µg/mL, while the amount leaked from the cells rose to 0.98 µg/mL after 30 min of contact time. At 60 min of contact time interval with this fraction, the amount of potassium leaked was 1.09 µg/mL. When the contact time was increased to 90 min, the quantity of potassium ion leaked from the cells was 1.14 µg/mL and this increased to 1.28 µg/mL after 120 min of contact time.

When the cells were exposed to the fraction at 2 x MIC concentration, the amount of potassium ions leaked out of the cells at 15 min was 0.99 µg/mL. At 30 min of contact time, the amount of potassium ion leaked from the cells increased to 1.01 µg/mL, with increased in the contact time to 60 min, the potassium ion leaked rose to 1.12 µg/mL. At 90 min contact time, the quantity of potassium ion leaked increased to 1.22 µg/mL and this increased to 1.38 µg/mL after 120 min of contact time. The effect of this fraction at 3 x MIC concentration followed the same trend as exhibited earlier on. The amount of potassium ion leaked from the cells at 15 min of contact time was 1.00 µg/mL and this rose to 1.22 µg/mL after 30 min of contact time. When the contact time was increased to 60 min, the quantity of potassium ion leaked increased to 1.36 µg/mL. At 90 min contact time, the quantity leaked out of the cells rose up to 1.41 µg/mL and 1.50 µg/mL after 120 min of contact time.

Each point represents the amount of potassium ion leaked (µg/mL) from the cells at a particular time interval in the presence of the fraction.

## The effect of ethyl acetate fraction on potassium leakage from *E. coli* cells

The potassium ion leaked from *E. coli* cells due to effect of ethyl acetate fraction at 1 x MIC, 2 x MIC, 3 x MIC concentrations indicated in **Figure 4**. At 1 x MIC concentrations in 15 min, the quantity of potassium ion leaked was 0.72 µg/mL, while this amount rose up to 0.73 µg/mL at 30 min interval. And with the increase in the contact time to 60 min, the potassium ion leaked was 0.76 µg/mL. There was an increase in potassium ion leaked from the cell at 60 min contact time to 0.82. Lastly, at 120 min of contact time, the quantity of potassium ion leaked rose to 0.89 µg/mL

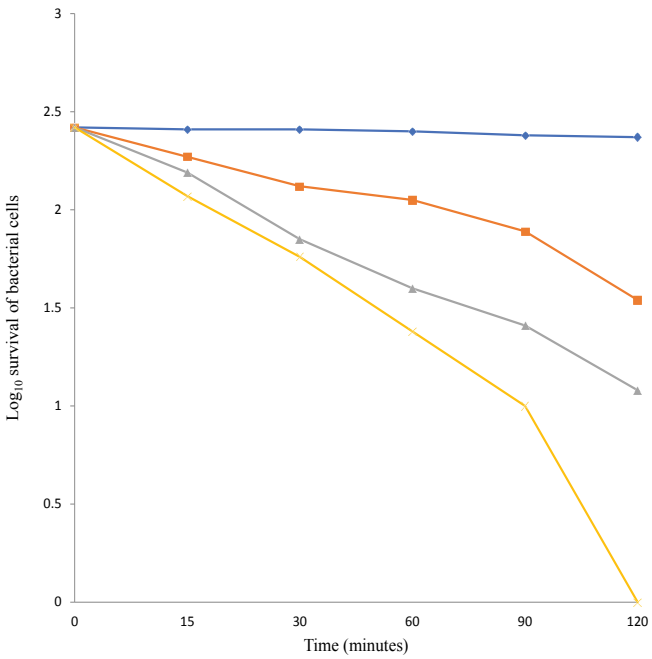


**Figure 1** The extent and rate of killing of *S. aureus* cells by ethyl acetate fraction at 1 x MIC (—■—), 2 x MIC (—▲—), 3 x MIC (—×—) and control (—◆—).

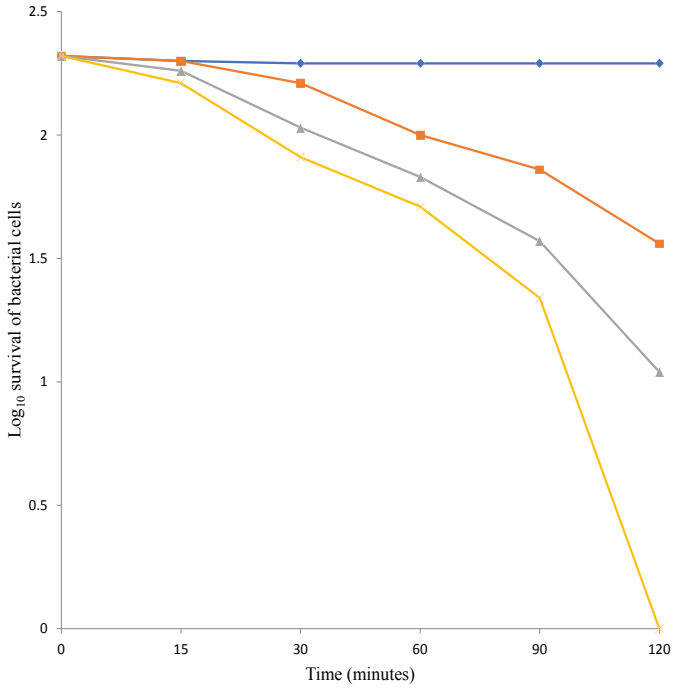


**Figure 2** The extent and rate of killing of *E. coli* cells by the ethyl acetate fraction at 1 x MIC (—■—), 2 x MIC (—▲—), 3 x MIC (—×—) and control (—◆—).

When the cells were exposed to the fraction at 2 x MIC concentration, the amount of potassium ion leaked out of the cells at 15 min contact time was 0.98 µg/mL. At 30 min of contact time, the amount leaked out from the cells increased to 1.03 µg/mL. The quantity leaked increase to 1.13 µg/mL at 60 min of contact time. When the contact time was increased to 90 min, the quantity of potassium ion leaked increased to 1.20 µg/mL and this got to 1,23 µg/mL after 120 min of contact time. The effect of this fraction at 3 x MIC concentration followed the same trend as previously described for other concentrations.

Each point represents the amount of potassium ion leaked (µg/mL) from the cells at a particular time interval in the presence of the fractions.

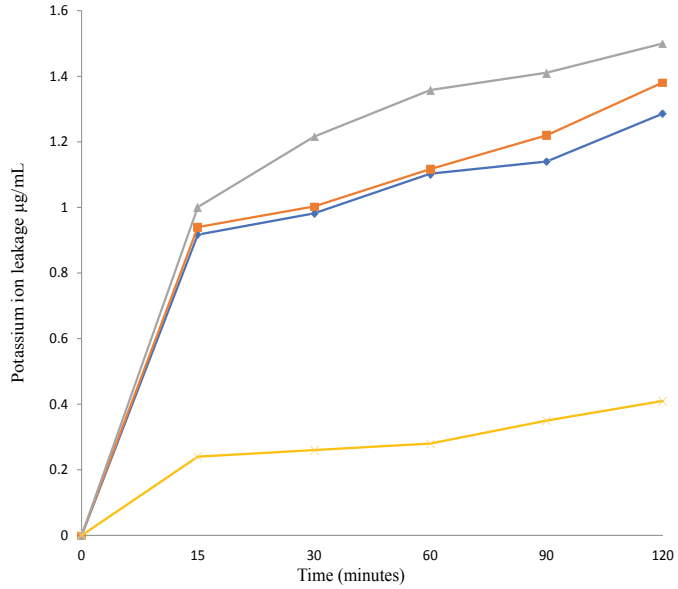


**Figure 3** The effect of ethyl acetate fraction on potassium ion leakage from *S. aureus* cells at concentration 1 x MIC (—◆—), 2 x MIC (—■—), 3 x MIC (—▲—) and control (—×—).

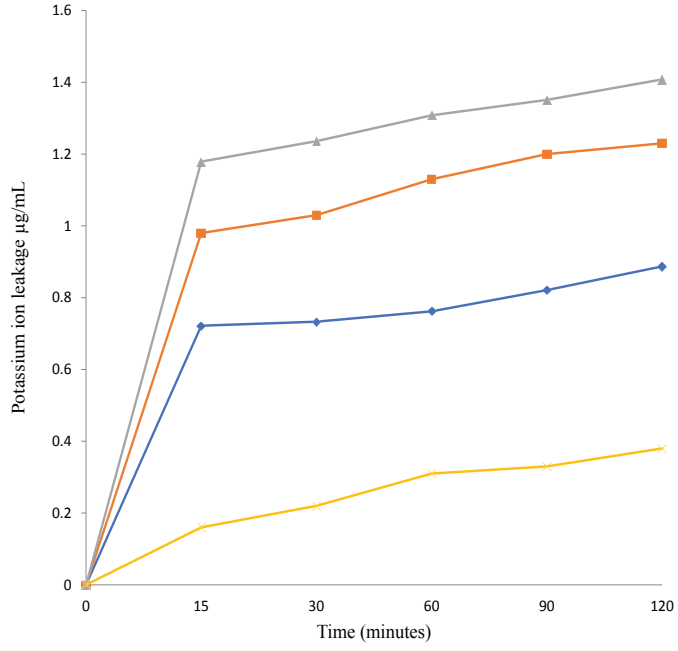


**Figure 4** The effect of ethyl acetate fraction on potassium ion leakage from *E. coli* at concentration 1 x MIC (—◆—), 2 x MIC (—■—), 3 x MIC (—▲—) and control (—×—).

## The effect of ethyl acetate fraction on nucleotide leakage from *S. aureus* cells

The nucleotide leakage from *S. aureus* cells due to the effect of ethyl acetate fraction at 1 x MIC, 2 x MIC, 3 x MIC concentrations indicated in **Figure 5**. The amount of nucleotide leaked from the cells at 1 x MIC concentration in 15 min was 1.33 µg/mL, while this amount rose up to 1.40 µg/mL after 30 min of contact time. At 60 min of contact time interval with this fraction, the amount of nucleotide leaked out was 1.47 µg/mL. When the contact time increased to 90 min, the quantity leaked out of the cells was 1.48 µg/mL and this increased to 1.53 µg/mL after 120 min of contact time. When the cells were exposed to the fraction at 2 x MIC concentration, the amount of nucleotide that leaked out of the cells at 15 min was 1.40 µg/mL. At 30 min of contact time, the amount of nucleotide leaked from the cells increased to 1.42 µg/mL, and at 60 min interval, the nucleotide leaked was 1.49 µg/mL. When the contact time was increased to 90 min, the quantity of nucleotide leaked increased to 1.58 µg/mL and this increased to 1.60 µg/mL after 120 min of contact time. The effect of this fraction at 3 x MIC concentration followed the same trend as exhibited earlier on with other concentrations. The amount of nucleotide leaked from the cells at 15 min of contact time was 1.47 µg/mL and this rose to 1.55 µg/mL after 30 min of contact time. When the contact time was increased to 60 min, the quantity of nucleotide leaked increased to 1.74 µg/mL, while it rose to 1.93 µg/mL at 90 min. Finally, the quantity of nucleotides leaked out of the test cells at 120 min contact time was 2.00 µg/mL.

Each point represents the amount of nucleotide leaked (µg/mL) from the cells at a particular time interval in the presence of the fraction.

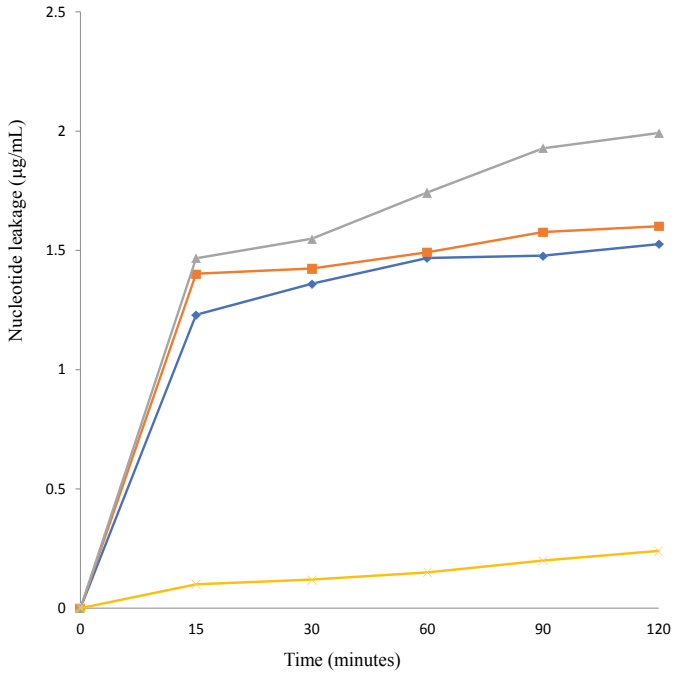


**Figure 5** The effect of ethyl acetate fraction on nucleotide leakage from *S. aureus* cells at concentration 1 x MIC (—◆—), 2 x MIC (—■—), 3 x MIC (—▲—) and control (—×—).

## The effect of ethyl acetate fraction on nucleotide leakage from *E. coli* cells

The nucleotide leakage from *E. coli* cells due to effect of ethyl acetate fraction at 1 x MIC, 2 x MIC, 3 x MIC concentrations indicated in **Figure 6**. The amount of nucleotide leaked from *E. coli* cells at 1 x MIC concentration in 15 min was 0.37 µg/mL, while the amount leaked from the cells at 30 min rose up to 0.42 µg/mL. At 60 min contact time, the nucleotide leaked out of the cells was 0.69 µg/mL. When the contact time was later increased to 90 min, the amount leaked out from the cells was 0.81 µg/mL. Lastly, at 120 min of contact time, the quantity of nucleotide leaked rose to 0.88 µg/mL.

When the cells were exposed to the fraction at 2 x MIC concentration, the amount of nucleotide leaked out of the cells at 15 min contact time was 0.98 µg/mL. At 30 min of contact time, the amount rose up to 1.03 µg/mL. The quantity leaked out of the cells increased to 1.13 µg/mL at 60 min of contact time. When the contact time reached 90 min, the quantity of nucleotide increased to 1.20 µg/mL and this got up to 1.23 µg/mL after 120 min of contact time. The effect of this fraction at 3 x MIC concentration followed the same trend as previously described for other concentration.

## The effect of ethyl acetate fraction on protein leakage from *S. aureus* cells

The protein leakage from *S. aureus* cells due to the effect of ethyl acetate fraction at 1 x MIC, 2 x MIC, 3 x MIC concentrations are shown in **Figure 7**. The amount of protein leaked from *S. aureus* cells at 1 x MIC concentrations in 15 min was 4.62 µg/mL, while the amount leaked from the cells rose to 6.04 µg/mL after 30 min of contact time. At 60 min of contact time interval with this fraction, the amount of protein leaked was 10.50 µg/mL. When

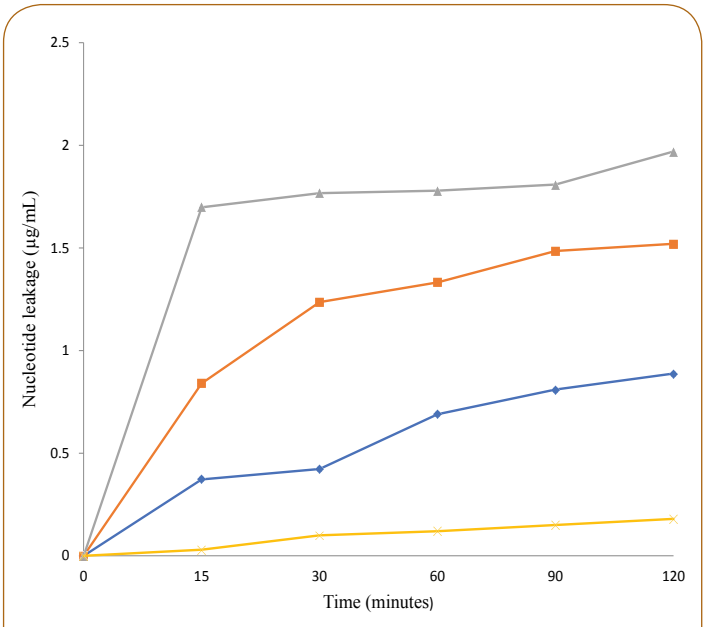


**Figure 6** The effect of ethyl acetate fraction on nucleotide leakage from *E. coli* at concentration 1 x MIC (—◆—), 2 x MIC (—■—), 3 x MIC (—▲—) and control (—×—).

the contact time increased to 90 min, the quantity of protein leaked from the cells was 11.42 µg/mL and this increased to 12.86 µg/mL after 120 min of contact time.

When the cells were exposed to the fraction at 2 x MIC concentration, the amount of protein that leaked out of the cells at 15 min was 9.04 µg/mL. At 30 min of contact time, the amount leaked from the cells increased to 9.28 µg/mL, with increased in the contact time to 60 min, the protein leaked was 11.62 µg/mL. When the contact time was increased to 90 min, the quantity of protein leaked increased to 12.85 µg/mL and this rose up to 15.58 µg/mL after 120 min of contact time. The effect of this fraction at 3 x MIC concentration followed the same trend as exhibited earlier on with other concentration. The amount of protein leaked from the cells at 15 min of contact time was 11.72 µg/mL and this rose to 16.79.

## The effect of ethyl acetate fraction on protein leakage from *E. coli* cells

The protein leakage from *E. coli* cells due to effect of ethyl acetate fraction at 1 x MIC, 2 x MIC, 3 x MIC concentration indicated in **Figure 8**. The amount of protein leaked from *E. coli* cells at 1 x MIC in 15 min was 4.28 µg/mL, while the amount leaked from the cells at 30 minrose up to 4.41 µg/mL. When the contact time was increased to 60 min, the protein out of the cells was 5.71 µg/mL. When the contact time was later increased to 90 min, the amount leaked from the cells was 6.90 µg/mL. Lastly, at 120 min of contact time, the quantity of protein leaked rose to 8.60 µg/mL.

When the cells were exposed to the fraction at 2 x MIC concentration, the amount of protein leaked out of the cells at 15 min contact time was 5.11 µg/mL. At 30 min of contact time, the quantity of protein leaked from the cells increased to 6.78 µg/mL. The quantity leaked increase to 7.44 µg/mL at 60 min of contact time. When the contact time was increased to 90 min, around 8.37 µg/mL of protein was leaked out of the cells and this got to 11.86 µg/mL after 120 min of contact time. The effect of this fraction at 3 x MIC followed the same trend as previously described for other concentrations.

## The antioxidant activity of *C. nucifera* endocarp extract

The *in vitro* antioxidant assay of *C. nucifera* reveals appreciable antioxidant potentials compared to ascorbic acid as the standard. The results of the antioxidant activity of *C. nucifera* indicated in **Figure 9**. Ascorbic acid used as the standard exhibited 50% inhibition ($IC_{50}$) at a concentration of 0.020 mg/mL. Highest percentage inhibition exhibited by *C. nucifera* endocarp extract was 79.80% at concentration of 0.015 mg/mL. The $IC_{50}$ of *C. nucifera* endocarp extract was found to be 0.011 mg/mL which is less than that of the standard ascorbic acid by 50%.

## The GC-MS analysis of partially purified ethyl acetate fraction of *C. nucifera* extract

The Gas Chromatogram-Mass Spectrometry chromatogram analysis of the partially purified ethyl acetate extract of *C.*

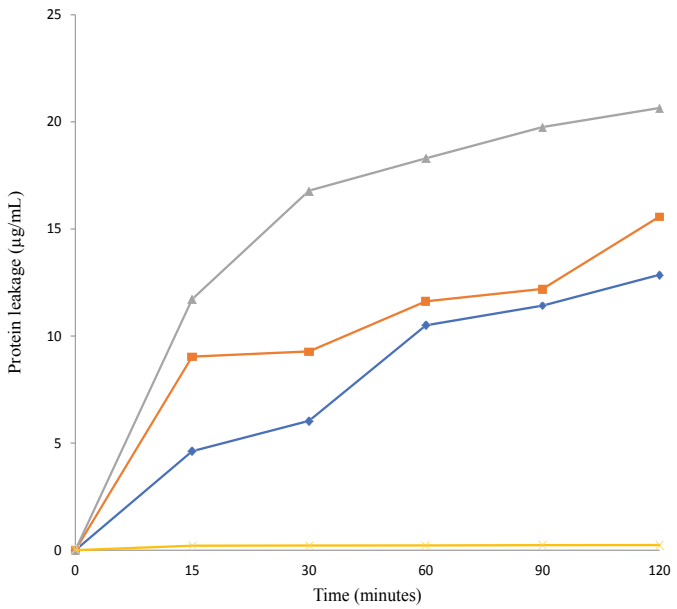


**Figure 7** **7:** The effect of ethyl acetate fraction on protein leakage from *S. aureus* cells at concentration 1 x MIC (—◆—), 2 x MIC (—■—), 3 x MIC (—▲—) and control (—×—).

**Note:** Each point represents the amount of protein leaked (µg/mL) from the cells at a particular time interval in the presence of the fraction.

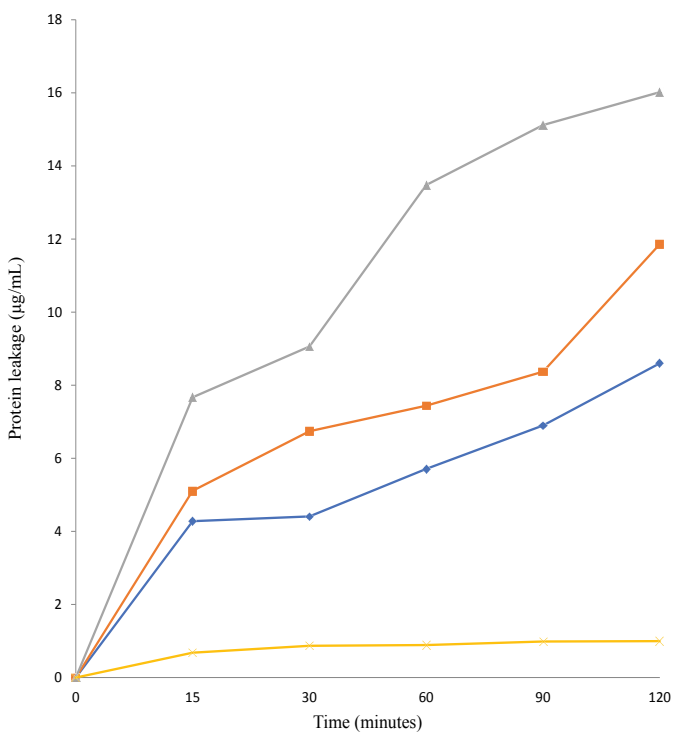


**Figure 8** The effect of ethyl acetate fraction on protein leakage from *E. coli* cells at concentration 1 x MIC (—◆—), 2 x MIC (—■—), 3 x MIC (—▲—) and control (—×—).

**Note:** Each point represents the amount of protein leaked (µg/mL) from the cells at a particular time interval in the presence of the fraction.

*nucifera* endocarp revealed seventeen peaks which indicated the presence of seventeen constituents (**Figure 10**). On comparison of the constituents mass spectra with National Institute Standard and Technology (NIST) 14.0 library, 2-methoxy-phenol, 2-Methoxy-4-vinylphenol, 2,6-dimethoxy-phenol, Ethyl-vanillin, 3-hydroxy-4-methoxy-benzadehyde, 2,4-di-tert-butylphenol, 2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl), Coniferyl aldehyde, 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol, 1-(2,5-Dimethoxyphenyl)-propanol, Hexadecanoic acid, 1-octadecene, Methyl 9-cis, 11-trans-octadecadienoate, 8-Octadecanoic acid, 1-Octadecene, 5-Eicosene, 1-Tetracosanol were identified to be present in the partially purified ethyl acetate fraction of *C. nucifera* endocarp extract. Of the seventeen compounds, the most prevailing compounds were Ethyl- vanillin with retention time of 8.996, 1- Octadecene with retention time 15.916 and n- Tetracosanol with retention time 19.525.

## Discussion

The crude extract at a concentration of 35 mg/mL inhibited the growth of nineteen out of twenty-five test strains (**Table 1**). On the other hand, the standard antibiotics; streptomycin and ampicillin used as positive controls inhibited eighteen and sixteen out of twenty-five bacterial strains tested respectively. The ethyl acetate fraction was the most active fraction exhibiting appreciable level of antibacterial activity against the test bacterial strains at a concentration of 10 mg/mL. This fraction inhibited nineteen bacterial strains while butanol and aqueous fractions inhibited six and three bacteria out of the twenty-five test organisms respectively at the same concentration of 10 mg/mL (**Table 2**). This is an indication that ethyl acetate would be a better solvent for extracting bioactive components of *C. nucifera* powdered endocarp. Antibacterial activities of partially purified samples obtained from ethyl acetate viz., Ethyl A, B, C and D also exhibited varying activities against the bacterial strains tested the findings which were in support of the work conducted by Adler et al. [23] in which *C. nucifera* extract was a pointer towards development of antimicrobial drug of natural origin to treat infections caused by some pathogens and in this case *S. agalaticae* known to cause neonatal sepsis and *Proteus mirabilis*, the causative agent of urinary tract infections which were also susceptible to the extract.

The minimum inhibitory concentration (MICs) and the minimum bactericidal concentration (MBCs) exhibited by the crude extract and ethyl acetate fraction were also investigated. The lowest MIC exhibited by the crude extract against the test bacterial strains was 0.27 mg/mL and the lowest MBC was 1.09 mg/mL (**Table 4**). On the other hand, the lowest MIC exhibited by the fraction was 0.31 mg/mL while the lowest MBC was 0.63 mg/mL (**Table 5**). According to Shanmughapriya et al., the MIC index of plant extract that is equal or less than 2mg/mL is considered to be bactericidal while those with the MICs above 2 mg/mL but less than 16 mg/mL, such extract could be taken as bacteriostatic [24]. Achinto and Munirudin concluded in their study that a low MIC value of medicinal plant extract indicates a better antibacterial activity [25]. This observation shows that extracts obtained from *C. nucifera* showed appreciable antibacterial activities having exhibited low MICs against pathogens used for this study. Thus this extract could serve as a good source of potent antimicrobial compounds or could be used synergistically with other synthetic antibiotics to combat gradual development of resistant to the existing antibiotics by some pathogens.

The medicinal values of plant lie in phytochemical compounds

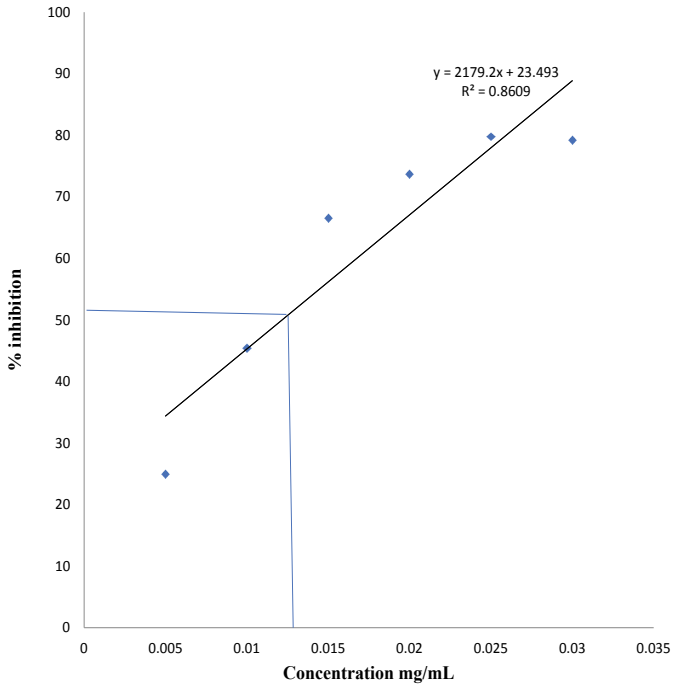


**Figure 9** Antioxidant activities of *C. nucifera*.

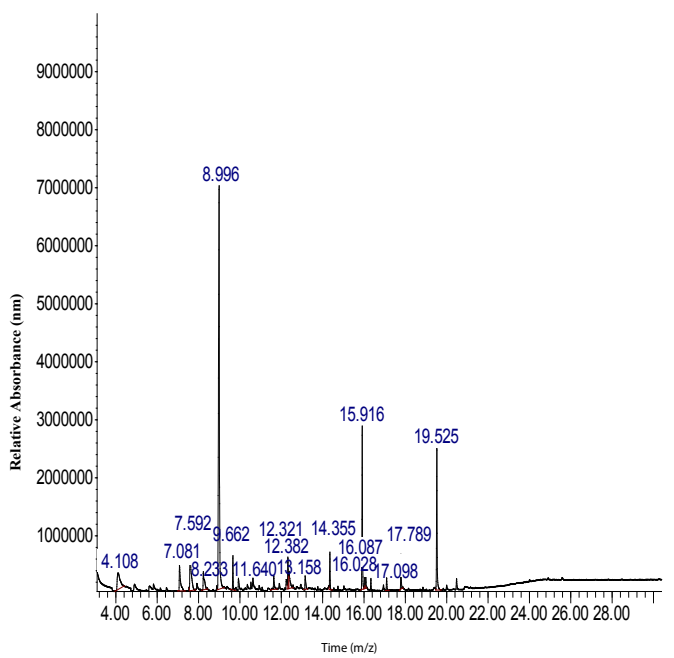


**Figure 10** GC-MS chromatogram of partially purified ethyl acetate fraction of *C. nucifera*.

which produced definite physiological actions on the human body [26]. This corroborated our findings as the phytochemicals revealed in the *C. nucifera* extract were alkaloids, flavonoids, phenols, triterpenes, saponins and tannins (**Table 6**). Phenolic compounds possess medicinal properties such as antiageing, anticarcinogenic, anti-inflammatory, anti-therosclerosis, cardiovascular protection and improvement of endothelial function as well as cell proliferation activities [27]. In addition, natural antioxidants mainly come from plant in form of phenolic compounds [28].

Scalbert, reported that Phenols present in *C. nucifera* extract could promote human health and reduce the effects of ageing and that flavonoids, one of the phytochemicals present in the extract of *C. nucifera* have the ability to scavenge hydroxyl and superoxide anion radicals which are important for diseases associated with oxidative damage of membranes, proteins and DNA [29]. Flavonoids may reduce risk of cancers, as well as preventing menopausal symptoms. Thus *C. nucifera* could serve as a major source of natural flavonoids that could be used to scavenge free radicals produced in human system. In our findings, existence of this phytochemical supports the usefulness of this plant in folklore medicine for preventing ageing in human. Saponins found in a wide range of plants existed in *C. nucifera* extract. Saponins are responsible for the most of the observed biological effects in medicinal plants [30]. Interactions of saponins with cell show lysing of the membrane [31]. Even low concentrations of some saponins have been reported by Eid et al. to apparently enhance the uptake of polar secondary metabolites, thus increasing their activity in an apparently synergistic fashion [32]. Saponins, along with other polar secondary metabolites in *C. nucifera* extract could have contributed to its antibacterial activity of this plant. Ukoha et al. reported tannins to be useful as an anti-inflammatory agent and treatment of burns and wounds and also tannin-rich remedies to be cytotoxic and antiparasitic [33]. Tannins are naturally occurring plant polyphenols and are found present in *C. nucifera* extract. They are able to form stable protein – tannin complexes and thus interact with wide variety of protein targets in microbes and its presence in *C. nucifera* extract confirmed the potency of the extract against test bacterial strains used for this study [31].

Alkaloids are another phytochemical compound present in the extract of *C. nucifera*. Alkaloids exhibit a wide range of biological activities which includes antibacterial, anti-asthmatic, anti-inflammatory and anti-anaphylactic [34,35]. All these confirm the therapeutic properties of this extract and therefore can be used for the treatment of several ailments caused by microorganisms.

Triterpenoids was also part of the phytochemical constituents observed to be present in *C. nucifera* extract. This compound has been reported to be cytotoxic against human larynx carcinoma and breast cancer [36]. The biological activities of this compound indicate that *C. nucifera* can be utilized to produce drugs for treatment of cancer and infections associated with various human pathogens. The antimicrobial activity of terpenoids which are lipophilic compounds has been associated with bacterial cell membrane disruption [37]. This might have contributed to the mode of actions of *C. nucifera* extract which led to the death of these organisms.

-*vitro* time kill assayare expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance of microrganisms to drugs [38]. The killing rates exhibited by ethyl acetate fraction against the test organisms increased with increase in concentrations and time intervals (Figures 1 and 2). For instance, the percentage reduction in cell viability of *S. aureus* after 15, 30, 60, 90 and120 min contact time with this fraction at 1 x MIC concentration were 30%,50%, 57.9%, 70.6% and 86.8% respectively. The percentage reduction in cell viability of *E. coli* at the same time and the same concentration were 5.1%, 24.1%, 47.6%, 65.1% and 82.5%. Potent bacterial killing was evident as extract concentration gets higher and time increases. This observation was similar to the antibacterial effect of *Hemidesmus indicus* reported by Saritha et al. [38]. The ability of this fraction to completely inhibit these pathogens at minimal contact time and at low concentrations is an indication that bioactive compounds in *C. nucifera* could be used for the development of antimicrobial compounds for treatment of infections caused by pathogens. Such antimicrobials could be useful in combating infections caused by multidrug resistant microorganisms.

In addition to killing rate exhibited by ethyl acetate fraction, other modes of action including leakage of potassium ions, proteins and nucleotides were studied. The fraction exhibited appreciable leakage of protein, potassium ions and nucleotides (Figures 3-8) which is an indication of bacterial cell membrane disruption by the antimicrobial compounds present in this extract. The ethyl acetate fraction exhibited appreciable potentials to cause potassium ion efflux from the test bacterial strains (Figures 3 and 4). The concentrations of potassium ions leaked from the test bacterial strains increased with increase in concentration of the fractions as well as increase in contact time of the cells with the fractions. The cytoplasmic membrane damage caused by antimicrobial agents could have caused the cations to be freely transported out of the cell and thus led to the death of the cell. Potassium ion is involved in the maintenance of a constant internal pH and membrane potential [39], therefore the efflux of potassium out of the membrane will have detrimental effect on the cell functions and lead to the cell death. The results obtained from this study showed that *C. nucifera* extract exerted its cidal effects on the test cells through disruption of their cell membranes. According to Zasloff, antibacterial agents could act by disrupting the cytoplasmic membrane of bacteria and as such cause destabilization and permeabilization [40]. This observation support the mode of action exhibited by ethyl acetate fraction obtained from *C. nucifera* crude extract. Spencer reported that many antimicrobial compounds that act on the bacterial cytoplasmic membrane induce the loss of 260 nm absorbing material and these were observed in the ethyl acetate fraction used against the test organisms [41]. The result obtained from this assay showed that the active fraction was able to cause leakage of nucleotides from the cells and thus loss of nucleotide through a damaged cytoplasmic membrane (**Figures 3 and 4**).

The protein leakage from the test microorganisms as observed in this study was proportional to the concentration of the fraction and the time of exposure of the cells to the solution of this fraction. From all indications, ethyl acetate fraction acts on the test cells and caused their death through cytoplasmic disruption as a result of increase in concentration and contact time. This is an indication of monophasic effect as reported by Akinpelu et al. [42].

The stability of DPPH free radical method is a sensitive way of determining the antioxidant activity of plant extract [43].Our findings showed that the extract of *C. nucifera* endocarp exhibited antioxidant activity which was found to be comparable with the ascorbic acid that was used as standard (Figure 9). *Cocosnucifera* extract significantly inhibited hydroxyl radicals produced by DPPH in this study and could serve as a free radical inhibitor or scavenger using its proton donating ability [44]. This study further supports the usefulness of this *C. nucifera* in scavenging hydroxyl radicals ($OH^-$) formed in the biological systems of humans which have been recognized as extremely damaging [45]. This plant can serve as a pointer towards development of antioxidant drug of natural origin. Such drug could go a long way in healthcare delivery.

In GC-MS analysis (Figure 10), major constituents of the most active partially purified fraction of the endocarp extract of *C. nucifera* was found to be Ethyl- vanillin, 1- Heniecosanol and 1- Tetracosanol, constituents of which greatly contributed to killing effects exhibited by *C. nucifera* extracts therefore useful in production of potent antimicrobial compound with broad spectrum activity.

## Author Contributions

All contributing authors have agreed to the submission of this manuscript for publication. DAA, MTA conceived and designed the study, performed the experiments, interpreted the results JOA helped to formulate the hypothesis in designing of project and wrote the paper. OOO and TJA monitored endocarp extract production experiment, analyzed the data, interpreted the results and edited the manuscripts.

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