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The Antimicrobial Property of the Acetone Extract of *Cola acuminata*

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Abstract

Cola acuminata extracts have been used for a variety of medical ailments including the treatment of microbial infections. Both methanolic and ethanolic extracts from *Cola acuminata* and *Cola nitida* has been shown to be effective against bacterial and fungal infections *in vitro*. However, the exact type of compounds and their effectiveness against specific bacteria has not been established. The aim of the current study was to purify a unique antibacterial activity from *Cola acuminata* that could be used to study the antimicrobial mechanism of action of Cola nut. We identified antimicrobial bioactive compounds present in *C. acuminata*, by sequentially extracting using solvents of increasing polarity, followed by SPE purification. The antimicrobial activity was evaluated using the agar well diffusion method and the MIC assay screened with *Staphylococcus aureus* as a model organism. We found the antimicrobial activity to be associated with the acetone (Biz-3) fraction and was specific towards gram-positive bacteria. An enriched antimicrobial activity, Biz-3w, was obtained using SPE (Biz-3w) fractionation which resulted in over a 60-fold purification of antimicrobial activity with a yield of 14µg/kg of Bizzy nut. We observed a dose-and time-dependent inhibition of *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis* with an effective dose ED50 of 4.3±0.03µg/mL, 3.04±0.08µg/mL and 6.72±0.03µg/mL, respectively. The MIC for Biz-3w in gram-positive organisms range from 14µg/mL to 16µg/mL and 30µg/mL to 50µg/mL in gram-negative organisms. The potency of Biz-3w relative to vancomycin was *Bacillus subtilis* ≥ *Staphylococcus aureus* > *Enterococcus faecalis* > *Klebsiella pneumoniae* > *Escherichia coli* with the gram-positive organisms being 5-6-times more sensitive towards Biz-3w. The result of this study points to a specific antimicrobial fraction with specificity towards gram-positive organisms and an enriched source for identifying the compounds responsible for this Bizzy nut antimicrobial properties.

Keywords: Antimicrobial; Bizzy nut; *Cola acuminata*; Natural products; Medicinal plants

Introduction

The growth of antimicrobial-resistant organisms has created a problem in the health service and food service industries. This has led to a search for an alternative treatment for diseases caused by certain human pathogens. In the past few years, several studies have been conducted on the antimicrobial properties of natural medicinal plants [1-3]. Many of these natural plants have been used because of their antimicrobial compounds that differ in their mechanism of action in comparison to current antimicrobial agents. These antimicrobial compounds present in plants are secondary metabolites of the plant which makes them potentially more effective against resistant microbial strains as compared to traditional antimicrobial agents [4,5]. Therefore, there is a need for new natural tools that can combat the spread of superbugs and minimize the use of chemical preservatives that can be detrimental to human health.

Cola acuminata and *Cola nitida*, are two eatable fruits from the family Sterculiaceae, that is indigenous to West Africa [6]. Its fruits contain seeds known as Cola nuts or Bizzy nut. The nuts are consumed by humans in different parts of the world because of its reported medicinal properties [1,7,8]. Cola nuts are used as a gesture of peace, friendship, hospitality, and it is important in various social ceremonies and religious activities of the Ebo people. It was transported to the Caribbean and South America where it is used in folk medicine [9].

Cola species are evergreen, mostly small or moderately sized trees, that are widely cultivated in tropical countries, including Jamaica and the Caribbean Islands [7]. The Bizzy nut is a "cure-all" herbal medicine that affects many biological processes. Bizzy nut is useful for many medical purposes, such as removal of poisons from the body; birth control; control of diabetes; weight loss; relief of menstrual cramps; possesses estrogenic and androgenic properties and contains antimicrobial activity [10-12]. Mubo reported that extracts of both *C. acuminata* and *C. nitida* contain antimicrobial and antifungal activity [8]. Sonibare

demonstrated that ethanol extracts of the leaves of *Cola acuminata* were found to be more effective against fungi than bacteria at high concentrations [5]. According to a report by Muhammad [12], an aqueous and methanol extract of the red and white variety of Cola nut showed antibacterial activity against the gram-positive bacterium, *Streptococcus anginosus*.

The antimicrobial potential of the two eatable forms of Cola nuts (*C. acuminata* and *C. nitida*) has been extensively studied but no systematic studies have been carried out to identify the active microbial inhibitory substance. There is however, inconsistency as to which type of extract (ethanolic, methanolic or aqueous) contains the bioactivity towards microorganisms. Furthermore, no study to our knowledge has identified the active ingredient responsible for Bizzy nut's antimicrobial properties. Thus, the objective of this study was to use the solid-liquid extraction of Bizzy nut coupled to SPE and HPLC purification to identify chemicals responsible for the antimicrobial activity. In this study, we demonstrate the presence of specific antimicrobial activity in the acetone extract of Cola nut, which has specificity towards gram-positive bacteria.

Materials and Methods

Plant materials

Cola acuminata seeds were obtained from the Lambs River, Jamaica. The plant tissues were cleaned, shade-dried, and powered by a mechanical blender.

Standard microorganisms

The test organisms used in this study were kindly provided by the Biology Department at Southern University, Baton Rouge and the streak plate method was performed to obtain isolated colonies and the purity of the organism ascertained using the gram stain technique. Organisms are designated as follows: Bacteria: *Bacillus subtilis* ATCC™ 1174™; *Staphylococcus aureus subsp. aureus* ATCC™ 33591™; *Enterococcus faecalis* ATCC™ 51299™; *Escherichia coli* ATCC™ 35218™; *Enterobacter aerogenes* ATCC™ 13048™; *Klebsiella pneumoniae subsp. pneumoniae* 700603™; *Pseudomonas aeruginosa* ATCC™ 27853™. Antibiotic and culture media were purchased from Fisher Scientific (Fisher Scientific, USA).

Preparation of *Cola acuminata* extracts

Finely ground Bizzy nut samples (100g) were sequentially extracted in a Soxhlet apparatus (120cm x 500cm) using 100% hexane, ether, acetone, methane, and water to produce five independent extracts with compounds of unique polarity. The extraction mixture refluxed for two days at temperatures corresponding to the boiling point of the respective solvent. Following extraction, particulate matter was removed by filtration, and the extracts were evaporated to dryness. All extracts were dissolved in 50% DMSO/PBS and represent the starting point for characterizing the bioactivity of Bizzy nut.

SPE purification of antimicrobial activity from Biz-3w

The acetone extract was adjusted to alkaline conditions of pH >10 by adding NH_4OH. The solution was added directly to a 60mL DSC-SCX SPE cartridge that was conditioned with 0.10M ammonium acetate/10% methanol, pH 4.5, at a flow rate of 2mL/min. The SPE cartridge was washed with 10 column volumes of conditioning buffer followed by elution of the sample with 50% and 100% methanol using a vacuum manifold set to a pressure of 2 mbar.

The DSC-SCX SPE wash fraction was loaded directly onto a SPE C18E column cartridge that was conditioned with 5% methanol. The SPE C18E column was eluted sequentially with 10 column volumes of 10%, 25%, 50%, and 100% methanol. Aliquots of each SPE fraction was filtered and evaporated to dryness. All samples were dissolved in 10% ethanol/PBS and stored at -20°C until use.

Antimicrobial turbidity activity assay

Antimicrobial activity was determined spectrophotometrically as the changes in absorbance at 650nm. The reaction mixture contained 100uL of PBS containing 0.1% ethanol and 0, 0.5, 5.0, or 50µL of sample. The sample was incubated for 3hrs or 6hrs at 30°C. The absorbance was read at 650nm and the activity expressed in U/mL. A standard curve was generated based on the prepared vancomycin standard solution (0.38, 0.19, 0.095, and 0.475mg/mL). One activity unit (AU) was defined as the amount of sample necessary to produce a decrease in cell viability equivalent to one microgram (µg) of vancomycin.

Determination of Minimum Inhibitory Concentration

The Minimum Inhibitory Concentrations (MICs) were determined by using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute [13,14]. To determine the antibacterial activities, two-fold serial dilutions of the compounds or standard antibiotics (0.0-64µg/mL) were prepared in Mueller-Hinton media (Merck, Darmstadt, Germany). Overnight bacteria stock inoculums were prepared by suspending a single colony of the examined microorganisms in 5mL sterile broth. A working suspension of bacteria was prepared by making a 1:2 dilution of the stock suspension until the turbidity of the inoculums was 0.5-McFarland standard as measured at 650nm wavelength. To each well of the microtiter plates containing 100uL of diluted extract, 0.1mL of the working inoculums were added, and the plates were incubated in a humid atmosphere at 37°C for 3, 6, 12, or 18 hours. Two hundred microliters of uninoculated media were included as a sterility control (blank) and 200µL of media with inoculums but without compounds were included as a growth control (positive control). The growth in each well was compared with that of the growth control well using the bacterial viability assay or turbidity assay at an absorbance of 450nm and 650nm, respectively. MICs were visually determined or from a plot of the percent viability versus compound concentration. MIC values were defined as the lowest

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concentration of the compounds producing 50% and 95% growth inhibition of the bacteria using the equation of the plot of viability or 650nm absorbance versus log of the concentration of the compound.

The Minimum Bactericidal Concentration (MBC) was determined by placing two microliters (2µL) of media obtained from each well of the 96-well plate of the overnight culture (from wells with bacteria showing no visible growth) onto a Mueller-Hinton agar plate (Merck, Darmstadt, Germany) or used to inoculate 100uL of Mueller-Hinton broth.

MBCs were determined as the lowest concentration yielding no visible growth (fewer than 4 colonies), which corresponds to a mortality of 99.99% of the microorganisms in the initial inoculums. Each experiment was performed in triplicates and the result reported as mean plus standard deviation.

Agar well diffusion method

The agar well diffusion method was followed to determine the antimicrobial activity. Two hundred and fifty microliters (250µL) of overnight culture with an OD corresponding to the 0.5-McFarland standard was spired over Mueller-Hinton agar plates containing 5mm diameter by 5mm depth wells. Five (5) mg/mL working solutions of each extract were prepared and 0-50µL added to separate wells of the agar plates and allowed to diffuse at 30°C overnight. Control experiments comprising of inoculums without plant extracts and inoculums with vancomycin discs were set up on separate agar plates. The diameter of the zone of inhibition (mm) was measured using a caliper tool and the activity index was also calculated. The experiment was performed in triplicates and repeated twice using different batches of extract. For each replicate sample, the zone of inhibition readings was taken in three different fixed directions and the average values were recorded.

Statistical analysis

All numerical data were expressed as mean ± standard error of mean (SEM). In each assay, three or four measurements were made. Means for the treatment groups were compared using analysis of variance and Duncan's multiple range test ($P<0.05$). To analyze the absorbance density from antimicrobial assays, a two-tailed t-test ($P<0.05$) was used to compare the mean (n=4) for each treatment group with the mean for the untreated control group. The GraphPad Prism 5.0 software program (San Diego CA) was used for the statistical analysis.

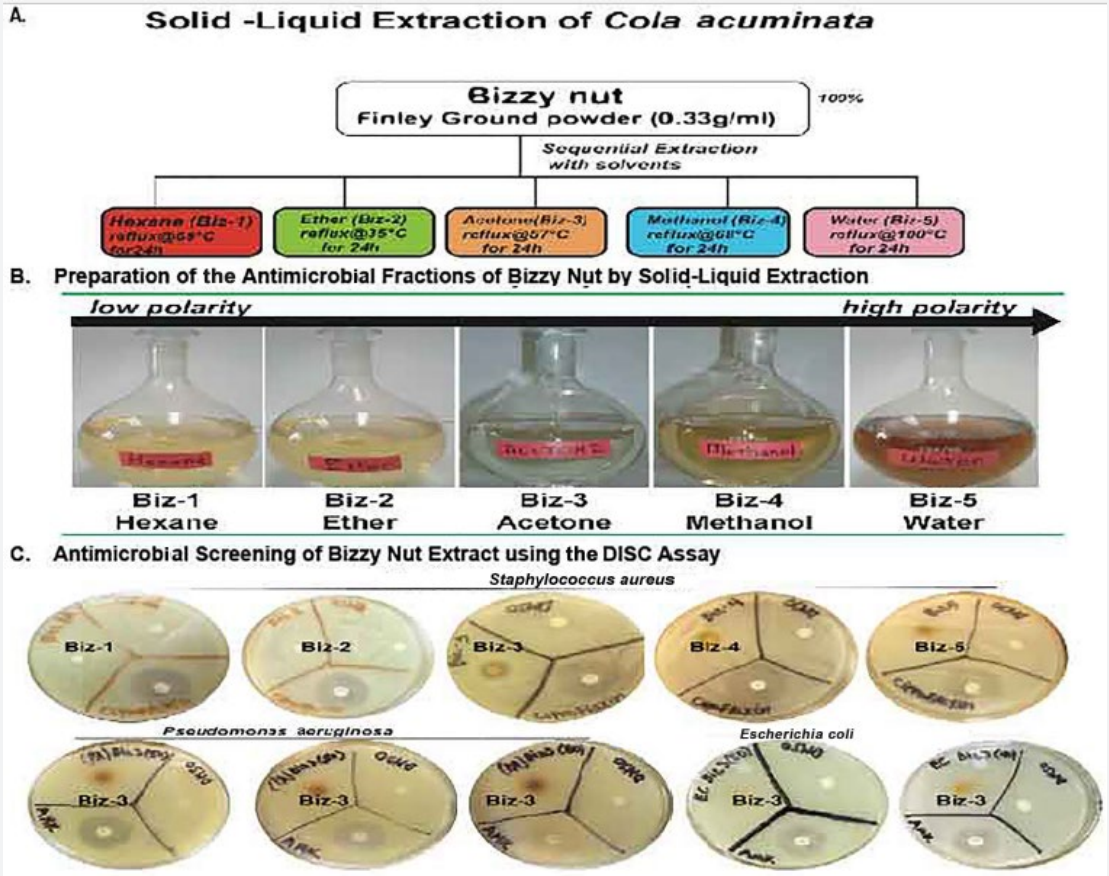

Figure 1: Isolation of the Antimicrobial Activity from *C. acuminata*. Bizzy nut was sequentially extracted with different solvents and the resulting analytes examined for antimicrobial activity. A. A schematic representation of the Soxhlet extraction. B. Different organic extracts, Biz-1, Hexane; Biz-2, Ether; Biz-3, Acetone; Biz-4, Methanol; and Biz-5, water. C. Disc Diffusion Assay using the different extracts from Bizzy nut.

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Results

Isolation of a unique antimicrobial bioactive fraction from *Cola acuminata*

There has been a rapid increase in microbial resistant bacteria which has triggered the need for new antimicrobial agents. Plant-derived products have been shown to be potential new sources for isolation and identifying antimicrobial agents. Thus, to identify new antimicrobial specific bioactive compounds present in *Cola acuminata*, we used solid-liquid extraction to divide the analytes present in *Cola acuminata* (referred to as Bizzy nut in this paper) into unique categories. Bizzy nut was sequentially extracted with solvents of increasing polarity to produce Biz-1, a hexane extract; Biz-2, an ether extract; Biz-3, an acetone extract; Biz-4, a methanol extract; and Biz-5, a water extract (Figure 1A & 1B) using a Soxhlet apparatus.

Each of the five extracts was screened for antimicrobial activity using *Staphylococcus aureus* as a model organism (Figure 1C). To determine which of the five extracts (Biz-1 to Biz-5) contains antimicrobial activity, the disc diffusion susceptibility test was performed. A confluent lawn of *Staphylococcus aureus* was created on Mueller-Hinton agar plates using an overnight culture containing an absorbance corresponding to the 0.5-McFarland standard. Filter discs containing 500µg of the extract were placed on the agar plates and the zone of inhibition formed at 30°C overnight. Figure 1C shows that of the five extracts tested, the acetone extract (Biz-3) produces the greatest degree of inhibition towards *Staphylococcus aureus* (zone equal to 15mm± 1.05). Examining the antimicrobial activity of the Biz-3 extract in *Pseudomonas aeruginosa* or *E. coli* (two gram-negative organisms) using the disc diffusion method showed no zone of inhibition at the concentration tested suggesting that the crude acetone extract (Biz-3) is specific to gram-positive organisms (Figure 1C).

Purification of the antimicrobial activity from the acetone extract of Cola nut

Since Biz-3 is being pursued as a potential source of novel antimicrobial drugs, an important issue is whether we can quantitate the levels of this antimicrobial activity present in Biz-3 extract. Therefore, we quantitated the amount of inhibitory activity in Biz-3, we examined the antimicrobial activity using three independent endpoints [15-17]. Figure 2 shows the inhibitory activity of Biz-3 using the agar well diffusion method, the turbidity assay, and the bacterial viability assay. All three assays produce an inhibitory response at a high concentration of Biz-3 using *Staphylococcus aureus* (<50µg/mL). We observed a linear relationship between growth-inhibition and Biz-3 concentration using the agar well diffusion method with a lower limit of detection of 5µg/mL. The turbidity assay was more sensitive in detecting the growth inhibition in the presence of Biz-3 as compared to the bacterial viability assay (GI50 turbidity 95%CI: 4.3-6.4µg/mL versus GI50 viability 95% CI: 9-12µg/mL). Although the turbidity assay and the bacterial viability assay measures different endpoints, they both resulted in a quantitative assessment of the antimicrobial activity in Bizzy nut and were used to monitor the purification of the antimicrobial activity from the Biz-3 extract.

Purification of antimicrobial activity of *Cola acuminata*

Next, we set out to purify and quantify the amount of antimicrobial activity present in Biz-3. We obtained an enriched antimicrobial fraction of the acetone extract, Biz-3, by coupling our solid-liquid extraction to SPE purification. The antimicrobial activity was purified directly from the acetone extract by adjusting the pH of the extract to 10 using NH_4OH before loading the sample on a DSC-SCX SPE cartridge. Three unique acetone fractions, SPE-Flow Through (Biz-3FT), SPE-Wash (Biz-3W) and SPE-Elutes (Biz-3E1-E3) were generated from the DSC-SCX SPE ion exchange column and screened for antimicrobial activity (Figure 3). The antimicrobial activity was excluded from the DSC-SCX SPE column and was concentrated in the 10% methanol wash (Biz-3w, fraction) (Figure 3A). The zone of inhibition produced by the SPE-Wash (Biz-3w) was 25mm comparable to that of 30ug of the vancomycin antibiotic disc (Figure 3B, panel 4 versus panel 8).

To assess the amount of antimicrobial activity present in Bizzy nut samples, we generated a standard inhibition curve using the turbidity assay and vancomycin as the standard antibiotic. We defined one inhibitory unit as an amount of sample necessary to produce a decrease in cell viability equivalent to 1.0ug of vancomycin (Figure 2D). The Bizzy nut fractions obtained from the solid-liquid extraction and those purified through a DSC-SCX ion exchange were assessed from the amount of inhibitory activity they contained (Figure 3B & Table 1). Each sample was concentrated using speed-vac, their weight in micrograms and, the amount of antimicrobial activity determined. The different volumes of the SPE fractions or equal weight of the solid-liquid extraction samples (Biz-1 to Biz-5) were assayed and the inhibitory units determined using the turbidity standard curve (Figure 2D & Figure 3B).

A summary of the antimicrobial purification is shown in Table 1. Using the turbidity assay, we observed that most of the antimicrobial activity was in the acetone (Biz-3) extract with a low amount of activity in the ether (Biz-2) extract. Both the turbidity and the disc-diffusion assay fail to detect antimicrobial activity in hexane (Biz-1), methanol (Biz-4) or water (Biz-5) extracts. The majority of the antimicrobial activity in the nut was seen in the acetone extract, Biz-3 (90% of the total activity). When ion-exchange chromatography was used to purify the inhibitory activity from the acetone extract, 84% of the inhibitory activity eluted in the 10% methanol wash (Biz-3w), whereas only 0.1% of the activity was present in any of the methanol eluted fractions (Table 1). The total activity of the Biz-3w fraction increased 60-fold with a specific activity of 1,612 units of inhibitory activity/mg of extract. The total activity of the FT was like the load (47,600 units) with a two-fold purification suggesting that the SPE

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column was saturated. Consequently, it appears that the level of antimicrobial activity was significantly concentrated by the SPE method and Biz-3w represents a significant source for further purification and characterization of the compound responsible for Bizzy nut antimicrobial properties.

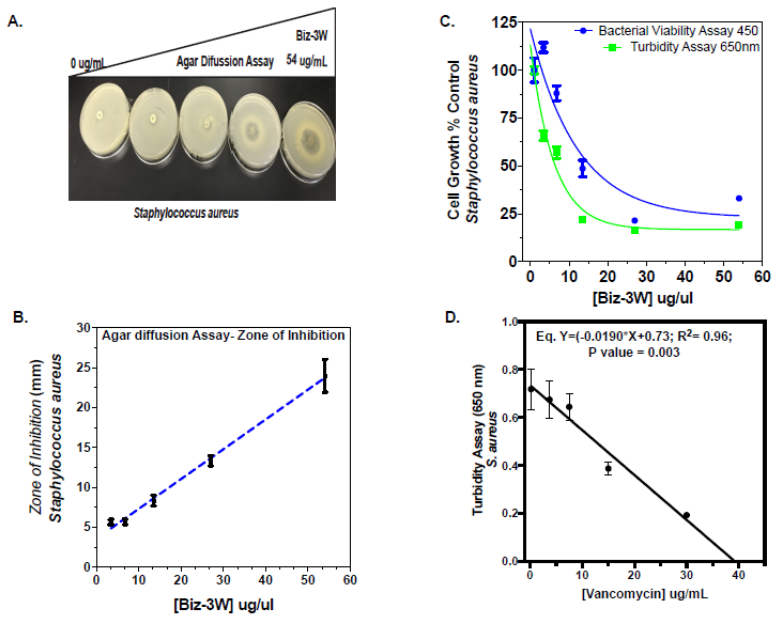

Figure 2: Standardization of the Antimicrobial Activity in Bizzy nut.
Antimicrobial activity of Biz-3w was assayed using the agar well diffusion assay (A-B); turbidity assay and the bacterial viability assay (C) using *Staphylococcus aureus* as the model organism. A standard curve of *Staphylococcus aureus* inhibition was generated using the turbidity assay and vancomycin as the standard antibiotic (D). One unit of inhibition was determined by linear regression and is defined as the amount of antibiotic or Biz-3 required to produce an OD 0.75 @650nm.

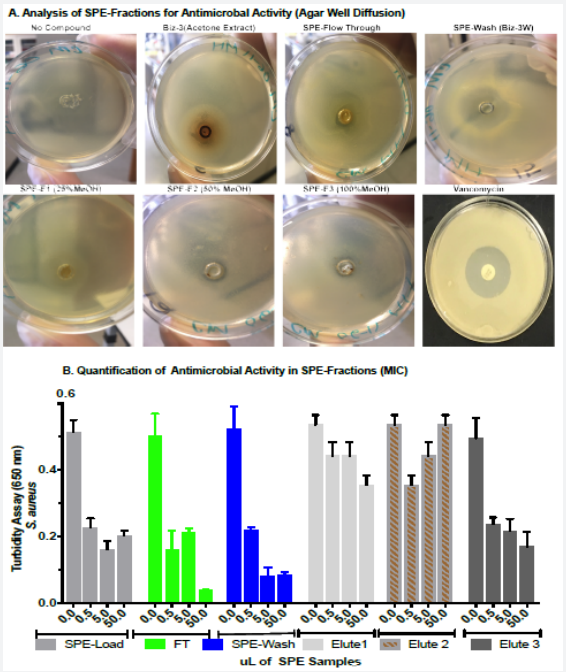

Figure 3: Purification of Antimicrobial Activity from Acetone Extraction.
The acetone extract (Biz-3) was acidified then purified through a DEC-SCX ion exchange SPE column. The bound sample was eluted using a different concentration of methanol and each sample assayed for antimicrobial activity. A. Agar well diffusion method using *Staphylococcus aureus*. B. Quantification of the Antimicrobial Activity in Biz-SPE-samples using the turbidity assay. The amount of inhibitory activity in each sample was determined using a vancomycin standard curve. One unit of activity is defined as the amount of antibiotic or Biz-3 required to produce an OD 1.0 @650nm.

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Table 1: Isolation of Biz-3w using Solid Phase Extraction.

Sample	Volume (mL)	Conc. (mg/mL)	Total Analyte (mg)	Yield mg/ kg Bizzy Nut	Unit/mL	Total Antimicrobial Activity	Specificity Activity Unit/mg	Fold-Purification	SPE-Yield (%)
Biz-3	325	5.34	1736	1	145	47,125	27.16	1	100
FT	350	2.48	868	0.5	136	47,600	54.84	2.02	100
Wash (Biz- 3W)	225	0.108	24.3	0.014	175	39,375	1,620	59.65	84
Elute 1									
SPE	100	0.016	1.6	1.00E-04	86	86	53.75	1.98	>0.18
Elute 2									
50% MeOH	100	1.7	170	0.098	67	67	0.39	>0.1	>0.18
Elute 3									
100% MeOH	100	2.57	257	0.19	0.84	84	0.32	>0.1	>0.18

The analyte concentration (mg/mL) of each fraction was determined gravimetrically from 2mL of the sample. Total analytes (mg) was obtained by multiplying the analyte concentration by the total volume of the sample. The activity, in units/mL, was obtained from the turbidity activity assay using vancomycin as a standard and the total units obtained by multiplying the activity by the total volume of the sample.
The specific activity (units/mg) was calculated by dividing the total activity by the total analytes in the sample. The fold-purification is a ratio of the specific activity of each sample to that of Biz-3. The yield (%) is a total activity (units) at each sample divided by the total activity (units) in Biz-3, multiplied by 100.

Effect of Biz-3w on gram-positive and gram-negative bacteria

Next, the growth inhibitory concentration and potency of Biz-3w towards gram-positive and gram-negative bacteria were examined. The susceptibility of Biz-3w extract was tested by the serial microdilution method and the agar well diffusion method in three gram-positive bacteria and three gram-negative bacteria. Figure 4 shows the morphologies of the organisms, the zone of inhibition, and the Minimum Inhibition Concentration assay for the gram-positive organisms.

The growth of all three gram-positive organisms in the presence of Biz-3w resulted in a dose-dependent inhibition after six hours. The calculated ED50 for Biz-3w was 4.3±0.03, 3.04±0.08 and 6.72±0.03µg/mL in *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis*, respectively. Biz-3w was much less effective in inhibiting the gram-negative bacteria tested (Figure 5). The ED50 for *Escherichia coli*, *Enterobacter aerogenes,* and *Klebsiella pneumoniae*, was 21.46±1.30, 25.00±1.26 and 20.45±0.13, respectively.

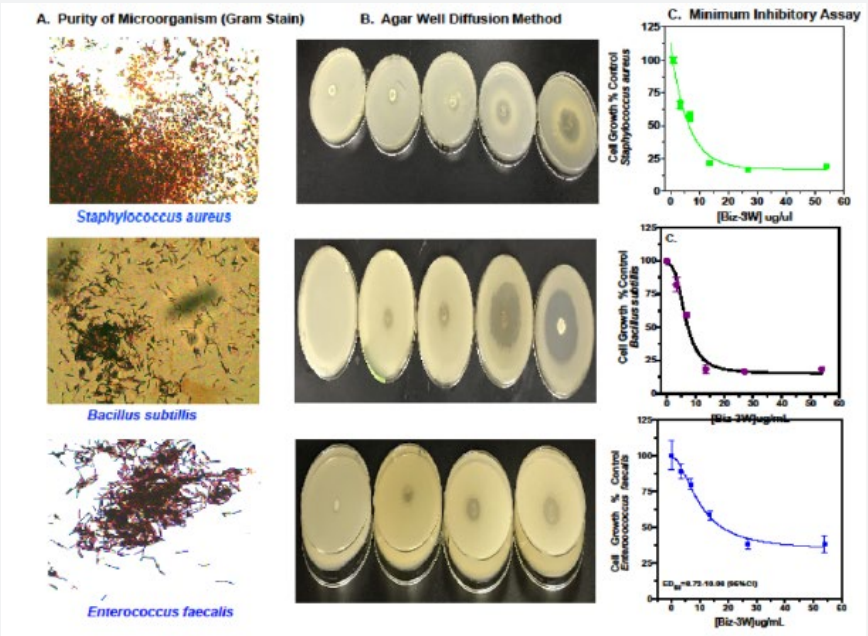

Figure 4: The Effect of Biz-3w on Gram-positive Organisms.
The identity of three different gram-positive organisms (*Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis*) was confirmed by gram staining (A) the antimicrobial activity of Biz-3w on these organisms was examined using the agar well diffusion method (B) and turbidity assay and MIC (C). The effective dose (ED50) of Biz-3w was determined by nonlinear regression of a plot of the percent inhibition versus Biz-3w concentration.

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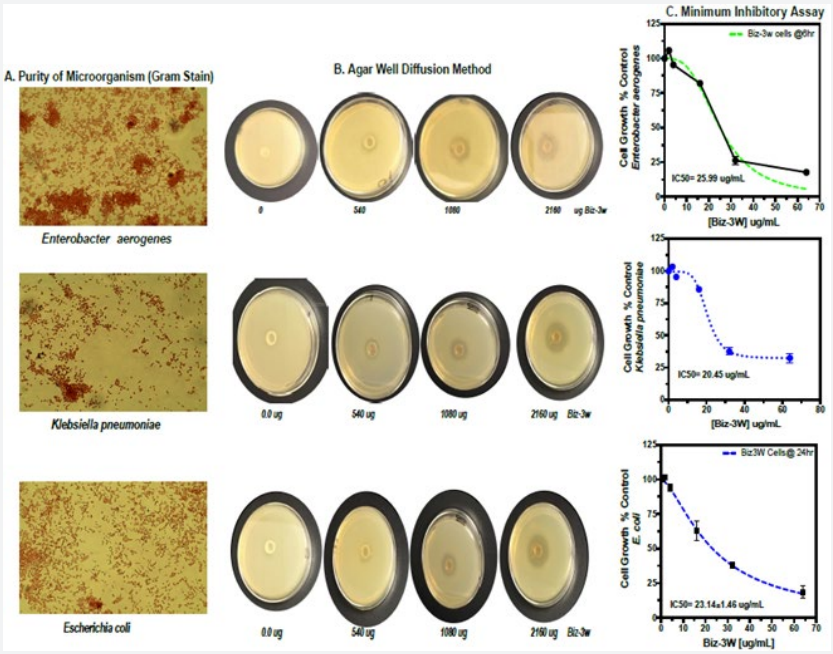

Figure 5: The Effect of Biz-3w on Gram-negative Organisms.
The identity of three different gram-negative organisms (*Enterobacter aerogenes, Klebsiella pneumoniae, Escherichia coli*) was confirmed by gram staining (A) the antimicrobial activity of Biz-3w on these organisms were examined using the agar well diffusion method (B) and the turbidity assay and MIC (C). The effective dose (ED50) of Biz-3w was determined by nonlinear regression of a plot of the percent inhibition versus Biz-3w concentration.

To determine the potency of Biz-3w relative to vancomycin, a dose-response curve was performed using a different concentration of Biz-3w and vancomycin. The Minimum Inhibitory Concentration (MIC) and Non-Inhibitory Concentration (NIC) were determined using nonlinear regression according to the method published by Lambert [18]. The growth inhibitory concentration of Biz-3w was determined by plotting the log of the concentration versus the percent growth (Figure 6). We defined the potency of Biz-3w in each organism as the ratio of the ED50 Biz-3w in test organism/ ED50 Biz-3w in *Staphylococcus aureus* with the potency in *Staphylococcus aureus* being set to one. Biz-3w inhibited the growth of all gram-positive bacteria at the concentrations of 1 to 20µg/mL and exhibited antibactericidal activity against all organisms. The NIC for Biz-3w was 1.09±0.06, 3.01±0.08; 4.47±0.0µg/mL for *Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis*, respectively. The MIC concentration of Biz-3w in *Enterococcus faecalis* was 37.15±0.23µg/mL, twice that of *Staphylococcus aureus* (MIC=16.53±0.23) and *Bacillus subtilis* (MIC=14.27±0.13) (Table 2). The order of potency of Biz-3w was *Bacillus subtilis* ≥ *Staphylococcus aureus* > *Enterococcus faecalis*.

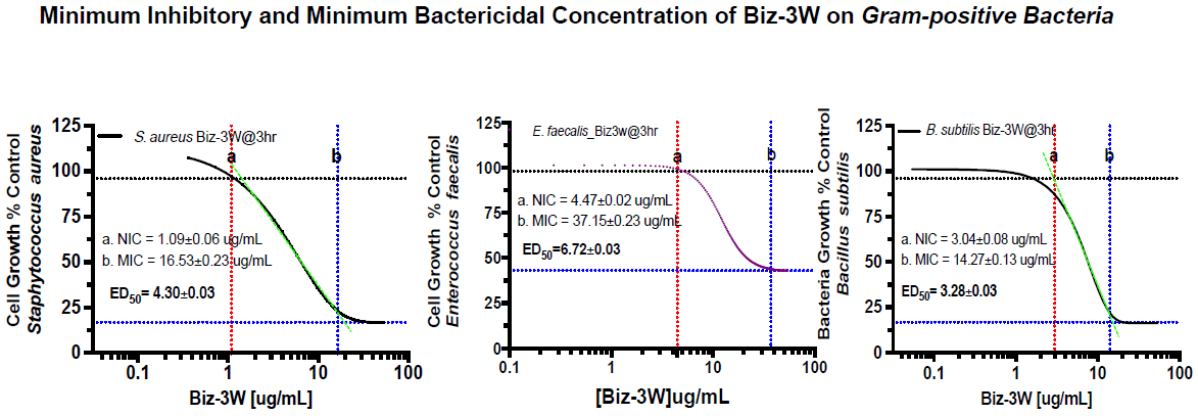

Figure 6: MIC and NIC analysis of Biz3w on *Staphylococcus aureus.*
The turbidity assay was performed using an increasing concentration of Biz-3w (0-50µg/mL) in the presence of *Staphylococcus aureus, Enterococcus faecalis* or *Bacillus subtilis* for 3hrs at 30°C. The resulting OD at 650nm was expressed as a percent of the control (0.0µg/ mL of Biz-3w) and plotted against the log of Biz-3w concentration. The NIC and MIC concentrations were determined by plotting the data to four parameters non-linear regression using GraphPad prism according to [18].

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Table 2: Potency of Biz-3w in Selective Microorganisms.

Strain	Description	IC50 (µg/mL)	MIC (µg/mL)	NIC (µg/mL)	Potency
Staphylococcus aureus	Gram-positive, non-motile, non-spore forming, aerobic, facultatively anaerobic organism. *Staphylococcus aureus* has been recognized as a cause of life-threatening infections such as bacteremia.	4.30±0.03	16.53±1.23	1.09±0.23	1
Bacillus subtilis	Gram-positive rod with the ability to produce endospores. It has been implicated in a wide range of infections including anthrax, abscesses, bacteremia/septicemia, wound and burn infections.	3.04±0.08	14.17±0.53	3.99±0.93	0.71
Enterococcus faecalis	Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. *Enterococcus faecalis* and *E. faecium* cause a variety of infections, including endocarditis, UTIs, prostatitis.	6.72±0.03	37.15±0.23	4.47±0.02	1.56
Escherichia coli	Gram-negative rod-shaped, non-spore forming bacterium. They are very common bacteria in the gastrointestinal tract and part of the normal bacterial flora.	21.46±1.30	53.58±0.13	5.75±0.03	5
Enterobacter aerogenes	Gram-negative rod-shaped bacterium generally found in the human gastrointestinal tract. It is a nosocomial and pathogenic bacterium that causes lower respiratory tract infections.	25.00±1.26	30.80±1.24	14.12±0.67	5.8
Klebsiella pneumoniae	Gram-negative, oxidase-negative, rod-shaped bacterium. *Klebsiella* can cause infections in the urinary tract, lower biliary tract, and surgical wound sites.	20.45±0.13	29.13±1.13	14.75±0.07	4.76

Effective dose (ED50), the Minimum Inhibitory Concentrations (MICs), and Non-Inhibitory Concentrations (NICs), were determined using the broth microdilution method as described in Materials and Methods. The IC50 was calculated by plotting the percent cell growth versus concentration of Bizzy to a non-linear variable slope model. The MIC (Minimum Inhibitory Concentration) and NIC (Non- Inhibitory Concentration) was calculated by the method of Lambert et al using GraphPad Prism. The potency of Biz-3w towards gram-positive bacteria and gram-negative bacteria was calculated by taking the ratio of the IC50 relative to *S.aureus.*

Discussion and Conclusion

Cola acuminata (Bizzy nut), possesses a variety of bioactive compounds which exhibit important bioactivities against the growth of certain mammalian cell lines, bacteria, and fungi. However, the specific chemicals responsible for these bioactivities have not been identified. Bizzy nut contains a myriad of active and inactive compounds (relative to antimicrobial properties) located in different parts of the plant cell. Therefore, solvents of different polarities were used to solvate the compounds and generate a fingerprint of the bioactive analytes present in Bizzy nut (Figure 1A). Using a selective solubility approach coupled to antimicrobial screening, we have identified a unique fraction of Bizzy nut (Biz-3w) which contains secondary metabolites capable of inhibiting gram-positive bacteria.

There is an increase in the number of antibiotic-resistant bacteria that is rendering the current antibiotics ineffective, thus fueling the need for new antibiotics that remain effective after continuous use. The antimicrobial activity identified in this study appears to target the gram-positive organisms. Our study demonstrates the inhibition of four of the top six multi-resistant pathogenic bacteria identified by the WHO (Table 2) [19,20]. Methicillin-resistant Staphylococcus aureus (MRSA) is recognized as a cause of life-threatening infections such as bacteremia, showed a susceptibility towards Biz-3w at a concentration of 4.3ug/mL with a MIC of 16.53ug/mL. On the other hand, *Klebsiella pneumoniae*, a bacterium responsible for healthcare-associated infections such as urinary tract and lower biliary tract, was insensitive to Biz-3w treatment. Our results demonstrate that Biz-3w was 5-6 times more potent towards gram-positive organisms, suggesting it may be a source for isolating new antibiotics for gram-positive bacteria.

Inhibition of most gram-positive microorganisms involves complex mechanisms linked to the inhibition of the synthesis of the cell wall, cell membrane, nucleic acids, and proteins as well as the inhibition of the metabolism of nucleic acids [4,8,21]. The antimicrobial activity identified in this study is as effective as vancomycin (Biz-3w ED50 of 4-5ug/mL versus vancomycin ED50 of 0.5-1.0ug/mL towards *Staphylococcus aureus*). This suggests that Biz-3w may be affecting membrane permeability or selectively inhibiting cell wall and ribonucleic acid synthesis of gram-positive organisms such as *Staphylococcus aureus* and *Bacillus subtilis* [21]. However, further work is needed to unravel the exact mechanism of action of Biz-3w in inhibiting gram-positive organisms.

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The search for an alternative treatment for diseases caused by certain human pathogens has given rise to an interest in research on plant extracts that inhibit microorganisms. Various extracts prepared from the roots, seeds or leaves of a variety of the Cola tree have been examined for antimicrobial activity. These Cola extracts were found to exhibit important inhibitory activities against the growth of selective bacteria and fungi [22]. Muhammad et al. [12] reported that an aqueous and methanol extract of the seed from red or white variety of Cola nut showed antibacterial activity against *Streptococcus anginosus*, a gram-positive bacterium, which is a member of the viridans streptococci. The antibacterial activities demonstrated by our study is in line with previous antimicrobial works of other species of Cola [8,16,11]. The result of this study, however, points to a specific antimicrobial fraction with specificity towards gram-positive organisms.

Our Biz-3w extract possesses significant inhibitory activity against tested pathogens supports the hypothesis that Cola nut can be used as a source in the development of new antimicrobial agents. Determination of the MIC is important in diagnostic laboratories because it helps in confirming the resistance of microorganisms to an antimicrobial agent and it monitors the activity of new antimicrobial agents. In the present study, the MIC values of Biz-3w obtained in this study were lower than the NIC values (Table 2, Figure 6) suggesting that the plant extracts were bacteriostatic at lower concentrations but bactericidal at higher concentrations. From the data obtained in this study, it is therefore worthy to mention that the acetone or the Biz-3w extract of Bizzy nut may be explored as a treatment of infections caused by *Staphylococcus aureus* or *Bacillus subtilis*.

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