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Antibacterial Assessment of Oils from Four Plants Against Selected Gentamicin-Resistant Gram-Positive Bacteria

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ABSTRACT

Background: The continuous rise of the menace of antibiotic resistance in microorganisms remains a global health problem, and this places a significant burden on the effective management of infections caused by multiple-resistant species. Phytocompounds are being constantly assessed for bioactive components to discover new products. This study was designed to investigate the antibacterial activity of oils from four plants - sweet almond (SAO), avocado (AO), black seed (BSO), and coconut (CO) against gentamicin-resistant Gram-positive bacteria from environmental sources.

Methods: Fifty-seven water and soil samples were collected, and gentamicin-resistant Gram-positive bacteria isolates were recovered and identified with the ABIS online Microbiology software. Antibiotic susceptibility testing was done using the Kirby-Bauer disc diffusion method and multiple antibiotic resistance indices (MARI) of isolates evaluated. The antibacterial activity of plant oils was evaluated with the agar well diffusion technique, and analyses of bioactive compounds in the oil samples using a gas chromatograph-mass spectrometer (GC-MS).

Results: Altogether, 60 Gram-positive Gentamicin-resistant bacterial isolates were recovered. The isolates spanned 34 species belonging to 11 genera, namely *Staphylococcus* spp. (27), *Bacillus* spp. (18), *Enterococcus* spp. (2), *Listeria* spp. (2), *Macrococcus* spp. (3), *Corynebacterium* sp. (1), *Lactobacillus* sp. (1), *Paenibacillus* sp. (1), *Rothia* sp. (1), *Salinicoccus* sp. (1) and *Streptococcus* spp. (3). A high proportion of the isolates were resistant to erythromycin and oxacillin at 96.7%, and ampicillin at 86.7%. Meropenem was observed to be the most effective *in-vitro*. All isolates (100.0%) exhibited multidrug resistance and had MARI above 0.4. In undiluted forms, plant oils exhibited very low inhibitory activity against isolates but improved upon dilution of the plant oils in the order BSO > SAO > CO > AO.

Conclusion: The extremely high levels of multidrug resistance suggest the rapid dissemination of antibiotic resistance traits in the community and are quite bothersome. The plant oils exhibited low antimicrobial activity, emphasising the need for a continuous search for bioactive compounds against multidrug-resistant pathogens. This study, therefore, recommends the *in-vivo* investigation of the plant oils and the possibility of a synergistic relationship of these plant oils with conventional antibiotics.

Keywords: Keywords: Gram-positive bacteria, Gentamicin-resistance, Black seed oil, Avocado oil, Coconut oil, Sweet almond oil

1. INTRODUCTION

Antimicrobial resistance has been a significant challenge for the last few decades. Antibiotics have been used effectively in treating various infections; however, as microorganisms evolve and acquire resistance, these drugs become ineffective against the target organisms. About 700,000 mortality cases have been reported worldwide due to drug-resistant microbial infections [1]. Murray et al. [2] reviewed the burden of antimicrobial resistance and estimated that about 4.95 million fatalities were linked to bacterial drug resistance in 2019. Many researchers have looked for alternatives to synthetic drugs by developing and applying natural antimicrobials to treat drug-resistant infections, among which we have plant essential oils.

Essential oils (EOs) are concentrated hydrophobic liquids and volatile secondary metabolites extracted from plants with their unique aromatic flavour, smell, and essence [3,4]. EOs are complex mixtures of volatile organic macromolecules, primarily terpenes, terpenoids, and phenylpropanoids, but they can also include oxides, sulfur derivatives, and fatty acids [4]. Plants use oils for various purposes in nature, including intra- and inter-species communication, insect and predator repellant and deterrent actions, pollinator attraction, seed germination inhibition, and antibacterial, antifungal, and wound healing activities [5]. EOs derived from medicinal and aromatic plants (MAPs) have antimicrobial properties in various industries, including pharmaceuticals, nutraceuticals, cosmetics, perfume, agronomy, and sanitary products [6]. Gram-positive bacteria are responsible for various infections in humans and animals. For instance, in severe cases, *Staphylococcus aureus* is responsible for skin and soft tissue infections and bloodstream infections. Similarly, *Listeria monocytogenes* is a widespread pathogen that causes severe foodborne illnesses and systemic disorders in animals and humans [7]. Gentamicin, an aminoglycoside, is a bactericidal agent inhibiting protein synthesis, and is active against most Gram-negative aerobes [8] and certain Gram-positive pathogens when combined synergistically with other antibiotic classes. It is particularly effective against many *Staphylococcus* spp. [9]. However, the aminoglycosides are toxic and can induce nephrotoxicity, ototoxicity, and neuromuscular blockade [8]. Given these, there is a need to seek less toxic alternative therapies against infections caused by these isolates.

Plant essential oils with their constituent chemicals have been known to exhibit antibacterial potential against several Gram-positive and Gram-negative bacteria and some other microorganisms [10]. These unique characteristics have been in researchers' spotlight worldwide. Moreover, compared to synthetic chemicals, EOs have a low level of toxicity, side effects, and chemical diversity in terms of activity [11].

Various methods have been used to explain the mechanism of action of EOs on microorganisms. EOs disrupt many cellular activities, including energy production (membrane-coupled), membrane transport, and other metabolic regulatory functions, by destabilising cellular architecture and increasing permeability [10]. Furthermore, the virulence of some bacterial strains is modulated by various essential oils through the inhibition of several processes such as biofilm formation, bacterial cell communication, expression of virulence genes, and quorum sensing [12].

A wide range of drugs currently in use are derived from nature (plants, animals or microorganisms) as advances in drug production are mainly from natural products. In the last four decades, roughly 75% of compounds with antibacterial activity used in clinical practices originated from natural sources [13]. Hence, this study aims to determine the antibacterial activity of four plant oils (sweet almond oil [SAO], avocado oil [AO], black seed oil (BSO), and coconut oil [CO]) against gentamicin-resistant Gram-positive bacteria from environmental sources, as well as to determine the antibiotic resistance patterns of the isolates, and the bioactive compounds in the oils.

2. MATERIALS AND METHODS

2.1 Study Locations

This study was undertaken at selected points within the Osogbo metropolis. Osogbo, the current capital city of Osun State with an area of 47 km^2, is found along coordinates 7.7827° N, 4.5418°E and has a projected population of 749,750 as of 2022 [14]. Each sampling point's global position system (GPS) coordinate was taken using a hand-held GARMIN GPS 72H (Taiwan) and GPS Essential® App. Arc-GIS software generated a map of the different sampling points, showing the study area (Figure 1).

2.2 Sample collection and processing

A total of 57 surface/swamp water and soil samples (comprising 27 water samples and 30 soil samples) were collected and assessed in the present study. The samples which were collected across different sites include sur-

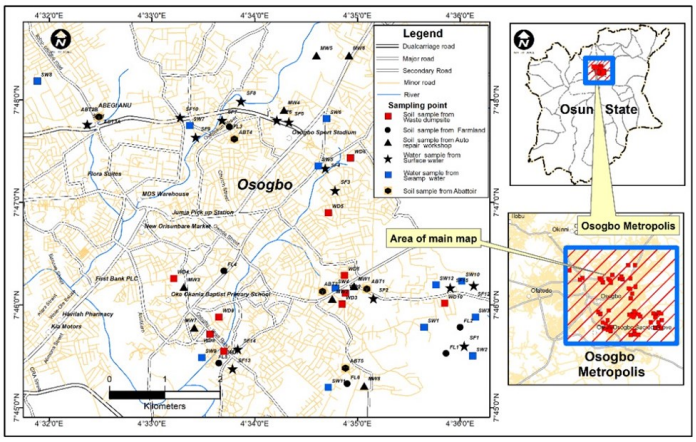


Figure 1: Map of the study area showing the various sampling locations

face water from streams (15 samples), swamps (12 samples) and soil samples from abattoirs (6 samples), automobile repair workshops (8 samples), farmlands (6 samples), and waste dump sites (10 samples) between January and September 2021. Water samples from streams and swamps were collected into sterile universal bottles by dipping the bottles a few inches below the surface of the water from selected points along the stream's length and then opening the bottle to allow the water to flow into the bottle. The caps of the bottles were then screwed tight while still underwater, removed and placed inside labelled individual Ziplock pouches. Soil samples were collected from the different sites via the conventional soil sampling technique. A 100g quantity of soil was collected from 25 –30cm depth into the ground. The soil samples were placed in sterile plastic Ziplock containers, sealed, and labelled. All samples were stored in coolers containing ice packs and were conveyed to the laboratory within 4 hours of collection for further processing.

A 1 ml aliquot of each water sample was inoculated into test tubes containing 9mls of Tryptone Soy Broth (TSB) supplemented with gentamicin at a concentration of 6mg/L and incubated for 48 hours at 37±2°C. For soil samples, 5 g of each sample was weighed into 50 ml of sterile distilled water in conical flasks and incubated overnight at 37°C in a shaker incubator. A 1 ml aliquot of the soil solution was then inoculated into TSB supplemented with gentamicin (6mg/L) and incubated for 48 hours at 37±2°C. Afterwards, the broth cultures were streaked on Tryptone Soy Agar (TSA) plates and incubated further for 24 hours at 37±2°C. Recovered isolates were Gram-stained, characterised using conventional biochemical tests, and the results were interpreted with the ABIS online Microbiology software for bacterial identification of the recovered isolates.

2.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done using the Kirby-Bauer disc diffusion method. Bacterial isolates were screened for sensitivity to commonly used antibiotics viz – ampicillin (10µg), gentamicin (30µg), erythromycin (15µg), cefoxitin (30µg), levofloxacin (1µg), meropenem (10µg), oxacillin (1µg) and vancomycin (5µg) (Oxoid, UK). Overnight bacterial cultures on Tryptone Soy Agar (TSA) were inoculated into sterile Ringer solution to give turbidity of 0.5 McFarland standard. Sterile cotton-tipped applicators, one per isolate, were used to inoculate sterile Mueller Hinton agar plates, creating a lawn of the pure isolate. An 8-place Oxoid disc dispenser was then used to set the discs aseptically on the seeded agar plates, and the plates were incubated at 37±2°C for 18-24 hours. Clear zones were examined visually after overnight incubation, and the diameter of each zone was recorded to the nearest millimetre. The interpretation was made using the EUCAST breakpoint table vs 11.0 [15], and the results were inferred as susceptible, intermediate, or resistant. Resistance to ≥ one drug in ≥ 3 antibiotic classes was used as the standard for classifying an isolate as multidrug-resistant, while Multiple Antibiotic Resistance Indices (MARI) of bacterial isolates were evaluated using established methods [16].

2.4 Plant oil sample collection and *in-vitro* antibacterial activity screening

Commercially available plant oils of sweet almond, avocado, black seed, and coconut of a standard and reliable brand were procured from a local supermarket and used for the screening in this study. The antibacterial activity of the plant oils was evaluated by the agar well diffusion technique on Mueller Hinton agar (MHA). A two-fold serial dilution of each plant oil was done using Dimethyl sulfoxide (DMSO) to give four different dilutions with final concentrations ranging from ½, ¼, $^1/_8$, and $^1/_{16}$, respectively.

An 18-to-24-hour culture of the test bacterial isolate was inoculated into 5ml of sterile Ringer solution, and turbidity adjusted to 0.5 McFarland standard, equivalent to approximately 10^6 CFU/mL. Seven wells of 6 mm diameter (at least 25 mm from each other) and at least 4 mm depth were then bored on prepared sterile Mueller–Hinton agar (MHA) plates with an appropriately sized sterile cork-borer. The agar surface was inoculated with the inoculum suspension to form a lawn by using a sterile cotton-tipped applicator to uniformly swab the entire surface of the agar. A 50µl aliquot of each original undiluted plant oil

and the four serial dilutions of each plant oil were dispensed aseptically into the bored wells on the inoculated plate with a micropipette and labelled accordingly. The plates were left on the bench at room temperature for an hour to allow the diffusion of the oil into the agar medium. The inoculated MHA plates were incubated in an upright position at 37±2°C for 18 – 24 hours and then examined visually for clear zones of inhibition around each well. The diameter of the zones of inhibition was measured to the nearest millimetre (mm) and recorded. Each test was duplicated, and the average value was evaluated and recorded. The absence of clear zones around the wells was reported as resistance. Chloramphenicol (50µl of 1 mg/mL) and DMSO (50µl each) served as positive and negative controls, respectively.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) analyses of plant oils

The analyses of the bioactive compounds in the plant oil samples were carried out using a Varian 3800/4000 gas chromatograph-mass spectrometer with electron impact (EI) as an ion source and equipped with an Agilent equipped with a capillary column DB5ms (30.0m x 0.25mm, 0.25µm film thickness). The EI ion source was set at 250°C and produced 70eV electrons with the GC column oven temperature programmed from 70^0C (hold time 2min) to 300°C (hold time 7min) at the rate of 10^0C min^{-1}. The total run time was 32.0 min. Nitrogen with 99.9995% purity was used as carrier gas with a constant flow of 1.51ml/min; 1 mL/min constant flow, and kept at a constant pressure of 95 kPa. The GC-MS interface temperature was 280 °C; injector and detector temperatures were set at 200 °C. A 1µl aliquot of the sample was injected in a split ratio of 1:10. The MS scan range was set from 40-800 Da. Identification of phytochemical compounds was obtained by using the database of the National Institute Standard and Technology MS library (NIST-MS library) by comparing the spectrum obtained through GC – MS to identify compounds present in the samples. No response factors were calculated. All the samples and replicates were continuously injected as one batch in random order to discriminate technical from biological variations. Additionally, the prepared pooled samples were used as quality controls (QCs), which were injected at regular intervals throughout the analytical run to provide data from which the repeatability can be assessed.

3. RESULTS

3.1 Frequency distribution of Gram-positive bacterial isolates among water and soil samples

Altogether, fifty-seven different water and soil samples were collected. A total of 60 Gram-positive bacterial isolates were recovered; 24 (40.0%) bacterial isolates from the water samples and 36 (60.0%) from soil samples, and these spanned 34 species belonging to 11 genera of *Staphylococcus* spp. (27), *Bacillus* spp. (18), *Enterococcus* spp. (2), *Listeria* spp. (2), *Macrococcus* spp. (3), *Corynebacterium* sp. (1), *Lactobacillus* sp. (1), *Paenibacillus* sp. (1), *Rothia* sp. (1), *Salinicoccus* sp. (1) and *Streptococcus* spp. (3) (Figure 2). The predicted accuracy of the bacterial species identification ranged between ≥ 80.20% and ≤ 99.0%. The predominant genera, *Staphylococcus* spp. constituted 45.0% of the total isolates and was recovered in all the study locations. *Bacillus* species followed this at 30.0%, recovered from all study sites except surface water. Bacteria isolated from the various sampling sites were 23.3% from swamp water, 18.3%

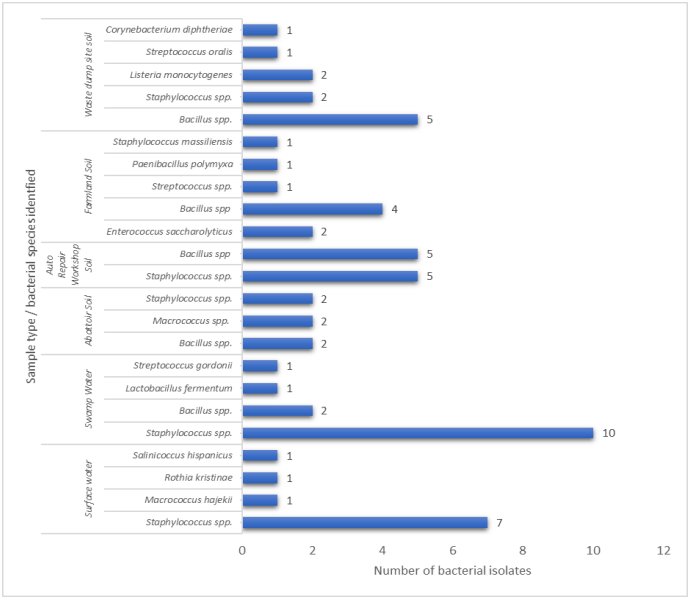


Figure 2: Frequency distribution of Gram-positive bacterial isolates among water and soil samples in Osogbo Metropolis.

from waste dump sites, 16.7% each from automobile repair workshops and surface water respectively, 15.0% from farmlands and 10.0% from abattoirs.

3.2 Antibiotic Susceptibility Testing of recovered Gram-positive bacterial isolates

The outcome of the screening for resistance to antibiotics is presented in Table 1. The results reveal that a high proportion of the bacterial isolates were resistant to erythro-

Table 1. Antibiotic Resistance profile of bacterial isolates obtained from environmental samples in Osogbo, Southwestern Nigeria.

Sample Type		Total[a]	Resistant Isolates; n (%)							
			AMP	GEN	ERY	FOX	LEV	MER	OXA	VAN
Water Sample	Surface Water	10 (16.7)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	9 (90.0)	2 (20.0)	10 (100.0)	10 (100.0)
	Swamp	14 (23.3)	14 (100.0)	7 (50.0)	12 (85.7)	7 (50.0)	11 (78.6)	0 (0.0)	14 (100.0)	8 (57.1)
Soil Samples	Abattoir	6 (10.0)	5 (83.3)	6 (100.0)	6 (100.0)	4 (66.7)	3 (50.0)	1 (16.7)	6 (100.0)	4 (66.7)
	Auto Repair Workshop	10 (16.7)	9 (90.0)	10 (100.0)	10 (100.0)	9 (90.0)	10 (100.0)	4 (40.0)	10 (100.0)	7 (70.0)
	Farmland	9 (15.0)	5 (55.6)	4 (44.4)	9 (100.0)	1 (11.1)	6 (66.7)	0 (0.0)	8 (88.9)	9 (100.0)
	Waste Dump Site	11 (18.3)	9 (81.8)	7 (63.6)	11 (100.0)	9 (81.8)	6 (54.5)	4 (36.4)	10 (90.9)	11 (100.0)
TOTAL		**60 (100)**	**52 (86.7)**	**44 (73.3)**	**58 (96.7)**	**40 (66.7)**	**45 (75.0)**	**11 (18.3)**	**58 (96.7)**	**49 (81.7)**

LEGEND: AMP = Ampicillin; **GEN** = Gentamicin; **ERY** = Erythromycin; **FOX** = Cefoxitin; **LEV**; Levofloxacin; **MER** = Meropenem; **OXA** = Oxacillin; **VAN** = Vancomycin; **Total[a]**= Total number of isolates tested

Table 2. Details of MAR Indices of recovered Gram-positive bacterial isolates by sample site

MAR index	WATER SAMPLES		SOIL SAMPLES				TOTAL (n = 60)
	SURFACE WATER (n = 10)	SWAMP (n = 14)	ABATTOIR (n = 6)	AUTO REPAIR WORKSHOP (n = 10)	FARM-LAND (n = 9)	WASTE DUMP SITE (n = 11)	
0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
0.1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
0.2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
0.3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
0.4	0 (0.0)	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.3)
0.5	0 (0.0)	3 (21.4)	0 (0.0)	0 (0.0)	5 (55.6)	1 (9.1)	9 (15.0)
0.6	0 (0.0)	3 (21.4)	3 (50.0)	0 (0.0)	2 (22.2)	2 (18.2)	10 (16.7)
0.7	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
0.8	1 (10.0)	2 (14.3)	1 (16.7)	2 (20.0)	2 (22.2)	3 (27.3)	11 (18.3)
0.9	7 (70.0)	4 (28.6)	2 (33.3)	7 (70.0)	0 (0.0)	5 (45.4)	25 (41.7)
1.0	2 (20.0)	0 (0.0)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	3 (5.0)

mycin and oxacillin at 96.7% (58 out of 60 isolates), followed by resistance to ampicillin (86.7%). Meropenem was observed to be the most effective *in-vitro* as only 11 out of 60 isolates tested were resistant. All isolates recovered from surface water were resistant to six out of eight antibiotics except only one isolate showing susceptibility to levofloxacin, while eight were susceptible to meropenem.

All recovered isolates (100.0%) were resistant to a minimum of three antibiotic classes; 81.7% (49 out of 60) were resistant to five or more antibiotic classes. Multiple Antibiotic Resistance Index (MARI) was calculated for each isolate, and the results are given in Table 2. The indices revealed that 100.0% of the isolates had MAR indices above 0.4, while 65.0% (39 out of 60) had MAR indices of ≥ 0.8.

3.3 Plant oil sample collection and in-vitro antibacterial activity screening

In the undiluted state, the plant oils exhibited a very low inhibitory activity against many of the bacterial isolates, the least active of the oils being AO. The pattern of activity was observed to be in the order BSO > CO > SAO > AO, with 35 (58.3%), 51 (85.0%), 53 (88.3%) and 58 (96.7%) out of 60 isolates being resistant to each plant oil respectively. However, the antibacterial activity improved slightly upon diluting the plant oils with DMSO as the degree of activity increased to some extent and the number of resistant isolates reduced. The pattern of activity was observed to be in the order BSO > SAO > CO > AO with 35 (58.3%), 37 (61.7%), 40 (66.7%) and 47 (78.3%) out of 60 isolates being resistant to all the five different dilutions of each plant oil respectively (Figure 3). Worthy of note is that there was no difference in the activity of BSO before and after dilution. Twelve isolates (20.0%) were completely resistant to all four plant oils, while seven (11.7%) were sensitive to all four plant oils. A detailed analysis of the antibiotic resistance patterns of the isolates against susceptibility to plant oils is given in Table 3.

3.4 Gas Chromatography-Mass Spectrometry (GC-MS) analyses of plant oils

Gas chromatography-mass spectrometry (GC-MS) analyses of the four plant oils uncovered various organic compounds. From the analyses, 20 different phytoconstit-

Table 3. Antibiotic Resistance and plant oil susceptibility pattern of bacterial isolates showing the different isolates recovered from environmental samples in Osogbo, Southwestern Nigeria

Isolates (No Tested)		ANTIBIOTICS (Number of Resistant Isolates)								PLANT OILS (No of Sensitive isolates)			
		Amp	Gen	Ery	Fox	Lev	Mer	Oxa	Van	SAO	AO	BSO	CO
Gram-positive cocci	*Staphylococcus haemolyticus (6)*	5	6	6	6	6	1	6	6	3	2	2	1
	Staphylococcus arlettae (4)	4	3	3	2	2	0	4	2	1	0	2	1
	Staphylococcus muscae (3)	3	2	3	1	3	0	3	2	0	0	0	1
	Staphylococcus massiliensis (2)	2	2	2	2	1	0	2	2	1	0	0	1
	Staphylococcus aureus (1)	1	0	1	1	0	1	1	1	1	1	1	1
	Staphylococcus cohnii (1)	1	0	1	1	1	0	1	1	0	0	0	0
	Staphylococcus equorum Subsp. Equorum (1)	1	1	1	1	1	0	1	1	1	0	0	0
	Staphylococcus gallinarum (1)	1	1	1	1	1	0	1	1	1	1	0	1
	Staphylococcus jettensis (1)	1	1	1	1	1	0	1	1	0	0	0	0
	Staphylococcus kloosii (1)	1	1	1	1	1	0	1	1	0	0	0	0
	Staphylococcus lutrae (1)	1	0	1	1	0	0	1	NT	1	0	1	0
	Staphylococcus saprophyticus (1)	0	1	1	1	0	0	1	1	0	0	1	1
	Staphylococcus vitulinus (1)	1	1	1	1	1	1	1	1	0	1	1	1
	Staphylococcus spp. (3)	3	3	3	2	3	1	3	3	3	2	1	2
	Streptococcus gordonii (1)	1	NT	1	NT	0	0	1	0	1	0	0	0
	Streptococcus oralis (1)	0	NT	1	1	1	0	0	1	1	0	1	1
	Streptococcus spp. (1)	NT	NT	1	NT	1	NT	1	1	0	1	0	0
	Enterococcus saccharolyticus (2)	2	1	2	0	1	0	1	2	2	0	1	0
	Rothia kristinae (1)	1	1	1	1	1	1	1	1	1	1	1	1
	Salinicoccus hispanicus (1)	1	1	1	1	1	0	1	1	0	0	1	1
Total	**Gram-positive cocci (34)**	**30**	**25**	**33**	**25**	**26**	**5**	**32**	**29**	**17**	**9**	**13**	**13**
Gram-positive rods	*Bacillus subtilis (3)*	3	4	3	4	3	0	3	4	3	2	4	2
	Bacillus mycoides (3)	2	2	2	1	2	1	3	2	1	0	2	0
	Bacillus anthracis (2)	1	NT	2	NT	2	0	2	2	2	0	1	0
	Bacillus licheniformis (2)	2	2	2	2	2	0	2	2	2	0	2	2
	Bacillus megaterium (2)	2	1	2	1	2	0	2	1	0	1	0	1
	Bacillus cereus (1)	1	1	1	1	1	0	1	1	1	1	1	1
	Bacillus pumilus (1)	1	1	1	1	1	0	1	1	0	0	0	0
	Bacillus spp. (2)	2	2	2	2	1	1	2	0	1	1	2	2
	Corynebacterium diphtheriae (1)	1	NT	1	1	0	1	1	1	1	1	1	1
	Listeria monocytogenes (2)	1	2	2	NT	NT	2	2	2	1	1	2	2
	Lactobacillus fermentum (1)	1	1	1	NT	1	0	1	0	0	0	0	1
	Macrococcus hajekii (2)	2	2	2	1	2	1	2	2	0	1	1	2
	Macrococcus brunensis (1)	1	1	1	1	1	0	1	1	0	0	0	1
	Paenibacillus polymyxa (1)	0	NT	1	NT	1	0	1	1	0	0	0	0
Total	**Gram-positive rods (26)**	**22**	**19**	**25**	**15**	**19**	**6**	**26**	**20**	**12**	**8**	**16**	**15**

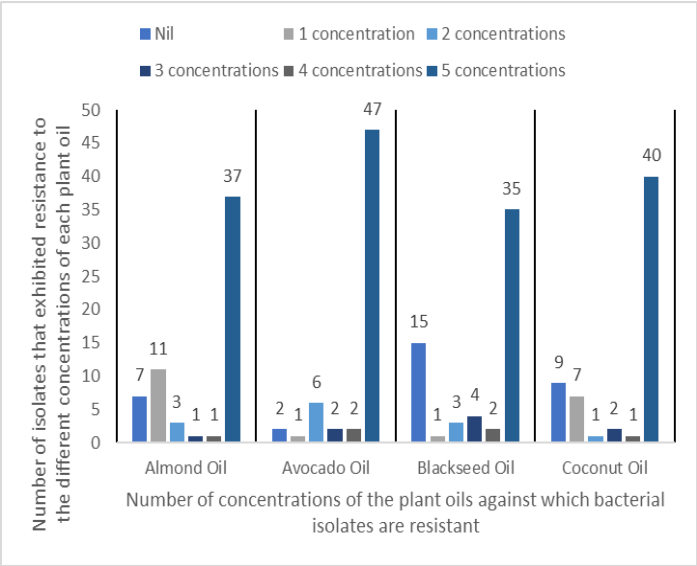
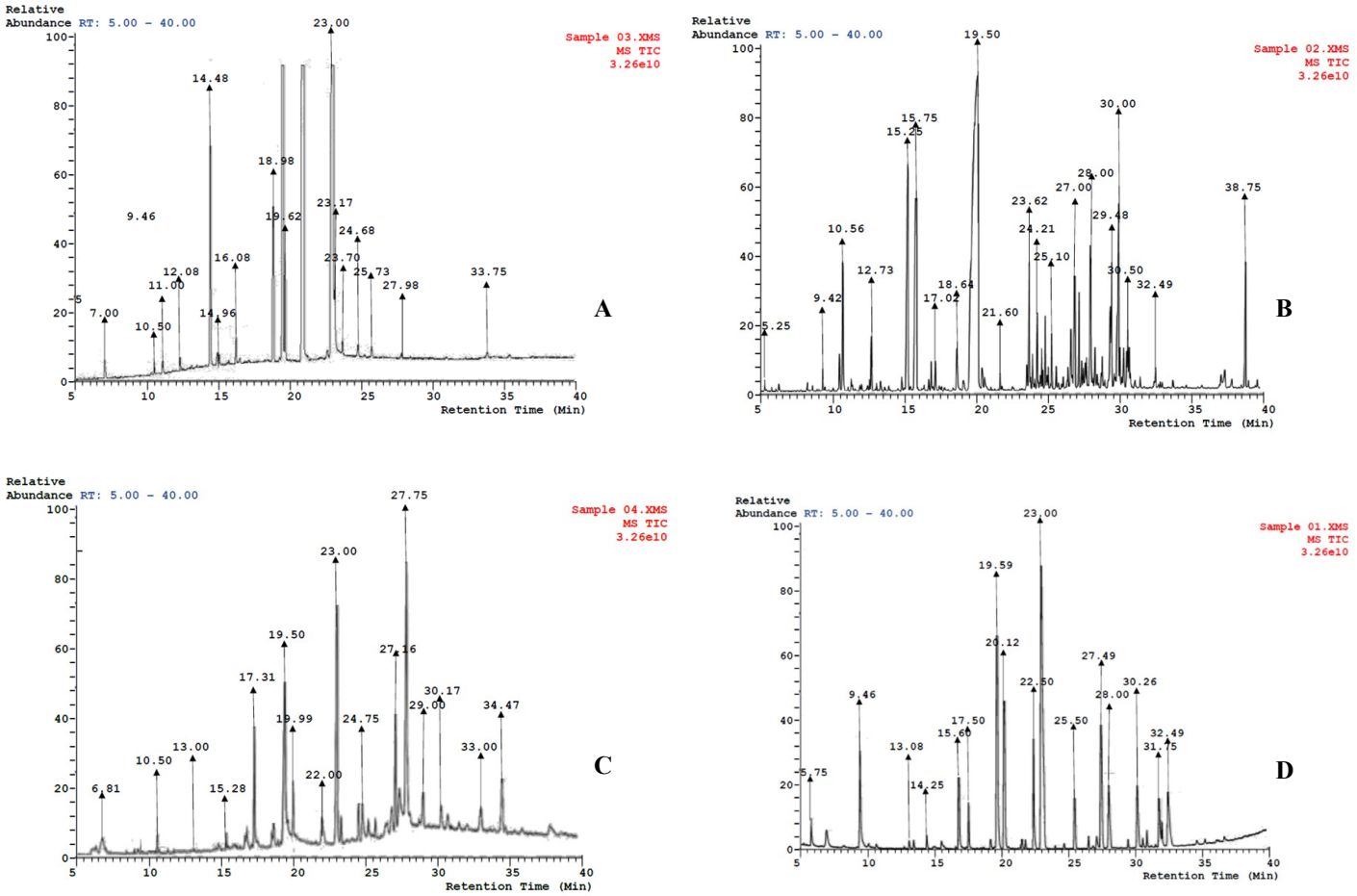


Figure 3. The Frequency of occurrence of resistant bacterial isolates to the different concentrations of the plant oils

uents were identified in AO, while the other three oils had 16 phytoconstituents each (Supplementary Figures 1a – 1d). The analysis reveals that none of the plant oils had precisely the same type and number of constituents; in fact, only a maximum of three constituents were found to be common to only three oils. Three fatty acid phytoconstituents - oleic acid, n-hexadecanoic acid, and 9,12-octadecadienoic acid (Z, Z) - were common to three of the plant oils (SAO, AO, and BSO), while hexadecanoic acid, ethyl ester was common to AO, BSO, and CO. The cyclic monoterpene - limonene was present in SAO and CO. Other constituents observed in the oils included, although not limited to alcohols, phenols, ketones, esters, carboxylic acids, furans, aldehydes, and pyrazine (Supplementary Table 1).



Supplementary Figures 1a – 1d. The chromatograms generated from GC-MS analyses of (a) almond oil, (b) avocado oil, (c) black seed oil, and (d) coconut oil

4. DISCUSSION

The goals of this study were to evaluate the possible antibacterial potentials of four plant oils, namely avocado, sweet almond, black seed, and coconut oils, against gentamicin-resistant Gram-positive bacteria recovered from environmental sources, to determine the antibiotic resistance patterns of the isolates, and to identify the phyto-components in the oils using GC-MS. The predominant bacterial genera were *Staphylococcus* (45.0%; isolated in all the study sites) and *Bacillus* (30.0%; recovered from all the study sites except surface water), comprising at least thirteen and seven different species, respectively.

The genus *Staphylococcus*, encompassing above 80 species and subspecies [17], belongs to the family Staphylococcaceae and the order Bacillales, an order which comprises other genera like *Bacillus, Enterococcus, Lactobacillus, Listeria, Macrococcus, Paenibacillus* and *Streptococcus* [18, 19], all of which representative species were recovered in this study; and the phylum Firmicutes. *Staphylococci* have reportedly been isolated from air and water environments, including wastewaters, industrial and hospital effluents [20], and soil [19, 21, 22]. *Staphylococcus* species are recurrently encountered in clinical and community settings. They are implicated in several infections in human hosts – ranging from skin, soft tissue, surgical site, wound, and bone infections to pneumonia, septicemia, and other potentially life-threatening infections [20]. They are highly infective agents of several hosts, including humans, animals (terrestrial and aquatic), and even plants [23].

Bacillus species have been reported from a wide range of ecosystems; they are common inhabitants of soil and aquatic sediments and are universally found in water, food, and soil [24]. Although a vast majority are considered non-pathogenic and generally regarded as safe (GRAS), *Bacillus* is an opportunistic pathogen capable of causing severe and life-threatening infections like septicemia and endophthalmitis if permitted entry into susceptible host tissues [25]. Some species are established human, livestock (*Bacillus anthracis* and *Bacillus cereus*), and insect pathogens (*Bacillus thuringiensis*), and all

Supplementary Table 1. Gas Chromatography-Mass Spectrometry (GC-MS) result showing organic components in Plant oil samples

PLANT OILS											
Almond Oil			Avocado Oil			Blackseed Oil			Coconut Oil		
Organic compound	Molecular Formular	Molecular Structure	Organic compound	Molecular Formular	Molecular Structure	Organic compound	Molecular Formular	Molecular Structure	Organic compound	Molecular Formular	Molecular Structure
Butanoic acid	$C_4H_8O_2$		Benzoic acid, 3,4,5-trihydroxy-	$C_7H_6O_5$		O-Cymene	$C_{10}H_{14}$		2H-Pyran-2-one, tetrahydro-6-methyl-	$C_6H_{10}O_2$	
DL-2,3-Butanediol	$C_4H_{10}O_2$		n-Decanoic acid	$C_{10}H_{20}O_2$		ß-Pinene	$C_{10}H_{16}$		Acetic acid	$C_2H_4O_2$	
Pentanoic acid	$C_5H_{10}O_2$		9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$		5Hydroxymethylfurfural	$C_6H_6O_3$		2-Octanol (internal standard)	$C_8H_{18}O$	
Benzaldehyde	C_7H_6O		Dodecanoic acid	$C_{12}H_{24}O_2$		D-Arabinose	$C_5H_{10}O_5$		n-Decanoic acid	$C_{10}H_{20}O_2$	
n-Hexadecanoic acid	$C_{18}H_{26}O$		n-Hexadecanoic acid	$C_{18}H_{26}O$		Oleic acid	$C_{18}H_{34}O_2$		δ-Dodecalactone	$C_{12}H_{22}O_2$	
2-Heptanol	$C_7H_{16}O$		Palmitoleic acid	$C_{16}H_{30}O_2$		Phenol, 5-methoxy-2,3,4-trimethyl-	$C_{10}H_{14}O_2$		Dodecanoic acid	$C_{12}H_{24}O_2$	
Oleic acid	$C_{18}H_{34}O_2$		Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$		p-tert. -Butylcatechol	$C_{10}H_{14}O_2$		Octanoic acid, ethyl ester	$C_{10}H_{20}O_2$	
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$		9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$		9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$		Acetamide, 2,2,2-trifluoro-	$C_2H_2F_3NO$	
9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$		9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$		9,12-Octadecadienoic acid, methyl Ester	$C_{19}H_{34}O_2$		2H-Pyran-2-one, tetrahydro-6-propyl-	$C_8H_{14}O_2$	
Acetic acid, pentyl ester	$C_7H_{14}O_2$		(2R,4R)-16-Heptadecene-1,2,4-triol	$C_{17}H_{34}O_3$		Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$		Limonene	$C_{10}H_{16}$	
Pyrazine, 2-ethyl-3,5-dimethyl-	$C_8H_{12}N_2$		Quercetin	$C_{15}H_{10}O_7$		Eicosanoic acid	$C_{20}H_{40}O_2$		Tetradecanoic acid	$C_{14}H_{28}O_2$	
Limonene	$C_{10}H_{16}$		Methyl stearate	$C_{19}H_{38}O_2$		β-Bisabolene	$C_{15}H_{24}$		Dodecanoic acid, ethyl ester	$C_{14}H_{28}O_2$	
2-Undecanone	$C_{11}H_{22}O$		oleic acid	$C_{18}H_{34}O_2$		cis-11,14-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$		2H-Pyran-2-one, tetrahydro-6-nonyl-	$C_{14}H_{26}O_2$	
Furan, 2-pentyl-	$C_9H_{14}O$		Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$		Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$		Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	
Tetracosanoic acid	$C_{24}H_{48}O_2$		Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$		Tetracosanoic acid	$C_{24}H_{48}O_2$		Methyl cholate	$C_{25}H_{42}O_5$	
			β-Sitosterol	$C_{29}H_{50}O$							
			Campesterol	$C_{28}H_{48}O$							
			Stigmastanol	$C_{29}H_{52}O$							
			Vitamin E	$C_{29}H_{50}O_2$							

three species have been reported to produce emetic toxins/enterotoxins [26].

Resistance to oxacillin and erythromycin was high at 96.7%, followed by resistance to ampicillin (86.7%). A total of 11 isolates were resistant to meropenem, showing meropenem to be the most effective *in-vitro* in this present study. All the 27 *Staphylococcus* species (100.0%) recovered in this study were resistant to oxacillin, 96.3% were resistant to erythromycin, while 92.6% exhibited resistance to ampicillin *in-vitro*. Only 4 (14.8%) were resistant to meropenem. Likewise, *Bacillus* species exhibited the same trend in resistance, showing resistance to oxacillin, erythromycin and ampicillin at 100.0%, 94.4%, and 88.9%, respectively; the least resistance was also to meropenem at 11.1% (2/18 isolates). The remaining species covering other genera were all resistant to erythromycin (100.0%), followed by oxacillin and vancomycin (86.7% each). It is important to note that a few isolates revealed breakpoint values that translated to sensitivity when screened against gentamicin despite having grown in the initial gentamicin-supplemented media used for culturing to screen for gentamicin resistance during isolation. The reason for this is not entirely apparent. Still, it may be due to possible variations in the concentration of the drug during screening and the disc, or maybe as a result of a loss in the property via horizontal gene transfer of extrachromosomal genetic material.

Resistance to multiple antibiotics has been reported in *Staphylococcus* species [27]. *Staphylococcus* species have acquired resistance mechanisms against virtually every antibiotic deployed against them, from β-lactams, aminoglycosides, vancomycin, fluoroquinolones, tetracyclines, and clindamycin, trimethoprim-sulfamethoxazole, daptomycin to linezolid [28]. These mechanisms include the production of β-lactamases, acquisition of antibiotic resistance genes (ARGs) by horizontal gene transfer, mutations in regulatory genes, formation of transmembrane cation channels, ribosomal methylation at the binding site of the antibiotics through efflux pumps, enzymatic inactivation or by a combination of more than one of these mechanisms [28].

Gentamicin-resistant *Bacillus* species in this study had high levels of resistance to oxacillin, erythromycin, and ampicillin at 100.0%, 94.4%, and 88.9%. This is in line with studies by other authors where resistance by *Bacillus* species to ampicillin was also high [11, 26], but is at variance with another study by Berić et al. [29], where only 3.0% of the isolate was resistant to gentamicin. In sharp contrast to the findings in this study is the report of susceptibility of the *B. cereus* group to erythromycin at 91.8% and gentamicin at 88.4% [26].

All the isolates (100.0%) in the present study were recovered from the environment and were multidrug-resistant, which is quite worrisome. None of them were susceptible to all the antibiotics against which they were screened, and they also had significantly high MAR indices - ≥ 0.4. Antimicrobial substances are employed in an eclectic array of environments, from the clinical setting, including veterinary outlets to farms, and this practice aggravates the scourge of antibiotic resistance development [30]. As reported by Ahmad and colleagues, many populations of multidrug-resistant pathogens and their resistance genes have been detected in myriads of samples from the environment [31]. Also, self-resistance in soil bacteria capable of antibiotic production and genes responsible for innate resistance in all or most non-producing environmental bacteria have been described [32]. Selective pressure brought about by human activities leads to the enhancement of antibiotic resistance determinants in bacterial populations. The transfer of these determinants to other bacterial strains, especially pathogenic bacteria and their expression under different conditions can be of public health concern [32].

Again, water, soil, and other natural environments offer a unique gene pool, harbouring potential genetic materials which could be attained and harnessed by pathogens to thwart antimicrobial activity [33, 34, 35]. Drivers of antibiotic resistance in soil and other environmental bacterial isolates include inappropriate/overuse of antibiotics, presence of heavy metals and biocides, as bacteria that carry metal resistance genes also frequently harbour antibiotic resistance genes, often present on mobile genetic elements [31, 36]. In contrast, biocides have been reported to facilitate the selection of antibiotic resistance in bacterial cells by mediating mutations [31, 37].

The pattern of activity of the diluted plant oils was observed to be in the order BSO > SAO > CO > AO. BSO was also the most active plant oil, even in the undiluted state. This corresponds with a previous study where BSO was reported to have strong antibacterial effects against *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus* [38]. This study, however, contrasts

sharply with the report of de Souza et al. [39], who found no antibacterial activity in the almond cake when screened against five bacterial strains. This could be a function of the plant part where the oil was extracted, the concentration of the oil, the technique, and the solvent employed in extraction [40]. As also observed in this study, CO was reported to possess an array of saturated acids and has been proven to have considerable antimicrobial activity against a wide range of organisms [41]. Another study also observed the inhibitory potential of CO on *Staphylococcus aureus* by the destruction of the bacterial cell walls [42].

Terpenes are polymers of isoprene (C_5H_8) and are classified into eight classes, including monoterpenes, based on the number of isoprene units present. This study revealed the presence of limonene, a cyclic monoterpene in SAO and CO, and O-Cymene, ß-Pinene, both monoterpenes and β-Bisabolene (sesquiterpenes) in BSO. Several studies have observed and reported the antibacterial activity of terpenoids, including mono-, di-, and triterpenes [43, 44]. The mechanism of antibacterial activity was found to correlate with the presence of hydroxyl groups, carbonylation of terpenoids as well as lipophilici-hydrophobicity [45].

BSO also contained phenols (Phenol, 5-methoxy-2,3,4-trimethyl- and p-tert. -Butylcatechol). Phenols are weakly acidic as they contain aromatic rings and the hydroxyl phenolic group, and a number of them are reported to have antibacterial activity like catechol. As reported by previous authors [46, 47], this activity is linked to the hydroxyl groups, as increase in antibacterial activity is observed with higher levels of hydroxylation [45]. The aromatic component is also suspected of playing a role [48]. Benzoic acid, 3,4,5-trihydroxy-, otherwise called gallic acid (a phenolic acid), was revealed in AO and has also been reported to be effective against a wide range of bacteria, including *Enterococcus faecalis, Pseudomonas aeruginosa, S. aureus, Moraxella catarrhalis, Streptococcus agalactiae, Campylobacter* spp*., Listeria monocytogenes*, and *Streptococcus pneumoniae* [49]. This was proposed due to its ability to alter cell wall integrity [50].

In conclusion, this study investigated the antibacterial activity of four plant oils against gentamicin-resistant Gram-positive bacteria from environmental sources. The extremely high levels of multidrug resistance, especially in environmental isolates, suggests the rapid dissemination of antibiotic resistance traits in the community and is quite bothersome. The plant oils exhibited a low antimicrobial activity against many isolated bacteria in the undiluted form despite previous reports of high antimicrobial potentials in three of them except avocado oil but improved upon dilution. The results reinforce the need for a continuous search for bioactive compounds against multidrug resistant pathogens from natural sources as the bane of multidrug-resistance in pathogenic bacteria rages on. This study, however recommends investigation of *in-vivo* assessment of the plant oils to ascertain the feasibility of attaining effective concentration *in vivo* and the possible synergistic relationships of these plant oils with conventional antibiotics.

Declaration of Conflict of Interest

The authors declare that no conflict of interest exists.

Authors' contributions

FMB Conceived and designed the analysis, **FMB, MKA, NY, ‡OOO** collected data, performed analysis, wrote the manuscript; **AAW, OOO, OHA, AAU** collected data, performed analysis. All authors approved the final version of the manuscript.

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