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Antibacterial Activity of Fixed Oils Extracted from *Annonamuricata* Leaves Against Antibiotic-Resistant Microbes

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Abstract: The growing prevalence of antibiotic resistant bacteria pose a significant challenge to the health globally. Although, there is need to source for alternative antimicrobial agents from natural product. This study evaluate the antibacterial efficacy of fixed oil extracted from *Annonamuricata*leaf against some selected bacteria (*Klebsiella* sp., *Pseudomonas* sp., *Escherichia coli,* and *Staphylococcus aureus*).Fixed oils were extracted using hexane solvent extraction and subsequently characterized using Gas Chromatography–Mass Spectrometry (GC–MS), which revealed the presence of bioactive compounds such as thymol, caryophyllene, hexadecanoic acid ethyl ester, and ethyl oleate.Remarkable areas of inhibition and concentration dependence of inhibition were observed against *Klebsiella* and *Pseudomonas* species. The values of MIC were 500 uL with *Klebsiell*a and 250 uL with *Pseudomonas* sp., showing a relatively strong antimicrobial activity. There was however no inhibitory effect against *Escherichia coli* implying resistance by nature. The oils proved to have bacteriostatic but not bactericidal activities against all the susceptible ones. Antibiotic susceptibility testing also established a multidrug resistance among the isolates especially to the commonly used antibiotics like cefuroxime, ceftazidime and amoxicillin. The present results indicate that *Annonamuricata* leaf fixed oils could serve as a potential source of antimicrobial agents that could be used by plants against some antibiotic-resistant bacteria. The research offers scientific grounds on the exploration of isolation, purification and use of these bioactive compounds in the designing of alternative therapeutic approaches.

Keywords: *Annonamuricata*, fixed oils, antibiotic resistance, antimicrobial activity, GC–MS, bioactive compounds

Introduction

Antibiotic resistant microorganisms have become one of the most urgent global public health issues of the twenty-first century due to their emergence and the spread within a short period (Salam et al., 2023). Historically, antibiotics have been very significant in the control of infectious diseases but their misuse and overuse in human medicine, in veterinary practice and agriculture has led to the emergence of resistant microbial strains (Ventola, 2015 World Health Organization [WHO], 2023). Consequently, a number of disease-causing bacteria have become resistant to widely-utilized antibiotics, limiting choices of treatment and contributing to a higher morbidity, fatality, and medical expenses on a global scale (Prestinaci et al., 2015). *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* are examples of antibiotic-resistant pathogens that have been gradually involved in hospital- and community-acquired infections, which pose acute challenges to the health systems of the world (Laxminarayan et al., 2013).

The growing epidemic of antimicrobial resistance has increased the effort to identify alternative antimicrobial agent's especially natural sources. Medical plants have been used as repositories of biologically active compounds that have a therapeutic potential. A large number of plant-based metabolites, such as alkaloids, flavonoids, terpenoids, and essential oils, have a potent antimicrobial activity and have been utilized in traditional medicine over the centuries (Cowan, 1999; Rios and Recio, 2005). These are natural compounds that are usually capable of acting in more than one way, which could diminish the probability of the microorganisms developing resistance apparently in contrast to the standard antibiotics (Bakkali et al., 2008). Plant-based bioactive compounds are thus being considered more with the view to being used as new sources of antimicrobial agents.

The *Annonamuricata* L. also known as soursop or graviola is one of the many medicinal plants that have received a significant amount of scientific interest because of its varied pharmacological effects. AnnonaceaeAnnonata, a tropical plant, is common in Africa, South America, and Southeast Asia and is also used in traditional medicine to dispel infection, inflammation, parasitic diseases, and other disorders extensively (Moghadamtousi et al., 2015). Past phytochemical researches have found out that the plant possesses numerous bioactive compounds, such as acetogenins, flavonoids, tannins, and alkaloids, which are linked with antimicrobial, antioxidant, anticancer, and anti-inflammatory effects (Gavamukulya et al., 2017; Coria-Tellez et al., 2018). In particular, the study of leaves of *A. muricata*has been extensively studied due to the high levels of these biologically active compounds in them. Plant leaves have fixed oils of fatty acids and lipid-soluble compounds in addition to secondary metabolites, which might have antimicrobial properties. Plant-based fixed oils have also been reported to disrupt the cell membranes of microbes, disrupt metabolism, and inhibit the growth of microbes (Burt, 2004). These properties render plant oils good sources to come up with new antimicrobial agents that can resist bacterial strains that are resistant. Although there are growing studies on the potential use of plant-derived oils as an antibacterial agent, little focus is made on the antimicrobial potential of fixed oils extracted directly out of the *Annonamuricata* leaves.

Though there are studies that have explored the antimicrobial property of the crude extracts and essential oils of *A. muricata,*(Abdul Wahab et al., 2018; Aguilar-Hernández) there is limited research on the use of the fixed oils extracted off the leaves of *A. muricata* as the antibacterial agent against antibiotic-resistant microbes. Moreover, currently available research highlights general antimicrobial screening but not one that targets clinically relevant antibiotic-resistant strains. The lack of information is a limitation to the knowledge of how we can use *A. muricata* leaf oils as alternative antimicrobial agents in the war against drug-resistant pathogens. Thus, the purpose of the present study is to determine the level of antibacterial efficacy of extraction of fixed oils of *Annonamuricata*leaves against chosen antibiotic-resistant microbial strains. The research aims to add to the increasing body of literature regarding the potential of natural products as alternative therapy in the management of antibiotic resistance infections by examining the inhibitory properties of these plant-derived oils.

Materials and Methods

Study area

The Study was carried out in the laboratory of Biochemistry and Biotechnology Department, Nile University of Nigeria, Abuja Nigeria

Sample collection

Fresh *Annonamuricata*(soursop) leaves were meticulously harvested from a farm located in KUJE LGA, Abuja, Nigeria. The plant was authenticated at the National Institute for Pharmaceutical Research and Development (NIPRD) utilizing herbarium reference specimens, and a specimen voucher number NIPRD/H/7321 was issued.

Sample Preparation

The leaves underwent a thorough washing process to eliminate any debris and contaminants, subsequently air-dried in a shaded environment at 40°C on a tray for a duration of one week (Neglo et al., 2021; Edet et al., 2022). During this period, they were covered with a mesh cloth to prevent nutrient degradation and were closely monitored to ensure complete desiccation. Following the drying process, the plant material was ground into a fine powder using a laboratory-grade blender.

Extraction of Fixed Oil from *Annonamuricata* Leaves

Fixed oil was extracted from the leaves of *Annonamuricata*with the aid of by employing a custom-fabricated apparatus. A solvent extraction process was carried out at SHETSCO (Sheda Science and Technology Complex, Abuja). 100g of dry desiccated soursop leaf powder wasused as the initial substrate. The powdered leaves underwent maceration in 300 milliliters of analytical-grade hexane, a non-polar solvent particularly suited for the extraction of hydrophobic compounds such as essential oils, fatty acids, and specific terpenoids. The maceration process extended over a duration of 24 hours, during which the mixture was intermittently agitated to ensure comprehensive interaction between the plant material and the solvent, thereby maximizing the yield of extractable lipophilic constituents.

After 24-hour, the mixture was filtered with aid of filter paper (Whatman No. 1 filter paper) to segregate the solid plant residue from the hexane extract. The filtrate was further subjected to solvent removal via a rotary evaporator. This technique adeptly evaporates the hexane under reduced pressure and regulated temperature, thereby preserving the integrity of heat-sensitive bioactive compounds. The resultant crude hexane extract was collected and meticulously stored in an amber vial at 4°C for subsequent phytochemical screening and antimicrobial activity assessment. (Álvarez-Martínez*et al.*, 2021)

Analysis of fixed oil obtained from Annonamuricata leaves using Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The mass spectrometer, interfaced with an Agilent GC/MS HP-5MS column measuring 30 m x 0.25 mm, featuring a film thickness of 0.25 mm, was employed to achieve the separation of compounds under a programmed temperature regime. This commenced at 59°C for a duration of 9 minutes, subsequently escalating to 230°C for 1 minute at a rate of 3 °C per minute, followed by a one-minute hold. The injector was maintained at a temperature of 245°C, while the flow rate of the carrier helium gas was set at 1 milliliter per minute. The ion source and analyzer for the mass spectrometry were operated at 260°C with an energy setting of 70 eV. The identification of the present compounds was accomplished through a comparative analysis of the spectral configurations obtained against the extensive mass spectral database

Collection of microbial strains

Clinical isolates of antibiotic-resistant pathogens were obtained from General Hospital Kuje, located in Abuja. The selected multi-drug resistant (MDR) strains included presumptively identified isolates of Methicillin-resistant Staphylococcus aureus (MRSA), Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli,Carbapenem-resistant Klebsiella pneumoniae,* and multi-drug resistant *Pseudomonas aeruginosa.* Identification was based on colony morphology and standard biochemical tests. To ensure the viability of these bacterial strains for ongoing research, they were maintained on nutrient agar slants at 4°C for short-term storage. For antimicrobial susceptibility testing, the isolates were subcultured on Mueller-Hinton agar plates, a standard medium recommended for antibiotic sensitivity assays.

Antimicrobial Susceptibility Testing Agar Disc Diffusion Method

In determining the antimicrobial activity of *A. muricata* fixed oils, we utilized the agar disc diffusion method was employed. 1000 µL of standardized bacterial suspension was uniformly distributed across Mueller-Hinton agar plates, with four inoculated plates prepared for each bacterial strain to ensure robust comparison (Irshad et al., 2012). Sterile paper discs, infused with varying concentrations of fixed oil (250, 500, 750, and 1000 µL), were strategically placed on the surface of each agar plate (Neglo*et al.,* 2021). Concurrently, multi-test antibiotic discs, encompassing a spectrum of standard antibiotics, were positioned on the opposing agar plates to serve as reference benchmarks for comparison (Neglo*et al.,* 2021). The plates were subsequently incubated at 37°C for a duration of 24 hours, after which the zones of inhibition surrounding each disc were meticulously measured to assess the antimicrobial efficacy of the fixed oils.

Results

Characterization of the fixed oils component of the leaves of *Annonamuricata* using appropriate extraction and analytical techniques.

The result of the GCMS analysis of fixed oils component of the leaves of *Annonamuricata* shows the present of compound such as Thymol, Cyclohexane, 1-ethynyl-1-methyl-2, Caryophyllene, Alpha- Caryophyllene, Napthalene 1,2,4a, 5, 6,8a-hexahyra, Napthalene 1,2,3, 5, 6,8a-hexahyra, Caryophyllene oxide, 1-(+)-Ascorbic acid, 2,6-dihexadicane, Hexadecanoic acid, ethyl ester, 9-Octadecanoic acid, methyl ester, V OCtadec-9-enoic acid, V Octadecanoic acid, V Ethyl oleate, V Ethyl 14-Methyl-hexadecanoate and Tetrapentacontane 1,54-dibromate (Table 1). The lowest retention time of the GC-MS analysis of fixed oils component of the leaves of *Annonamuricata* shows that Thymol has the lowest retention time of 6.50 while

Tetrapentacontane 1,54-dibromate has the highest retention time of 17.11 (Figure 4.1)

Table 1: GC-MS analysis of fixed oils component of the leaves of *Annonamuricata*

Peak	Retention time	Area	Height (%)	Compound
1	6.50	1.44	2.18	Thymol
2	7.60	4.16	5.86	Cyclohexane, 1-ethynyl-1-methyl-2
3	7.92	12.03	16.31	Caryophyllene
4	8.23	1.45	2.12	Alpha- Caryophyllene
5	8.60	3.26	4.17	Napthalene 1,2,4a, 5, 6,8a-hexahyra
6	8.80	3.84	5.69	Napthalene 1,2,3, 5, 6,8a-hexahyra
7	9.48	1.56	2.00	Caryophyllene oxide
8	14.37	19.88	18.05	1-(+)-Ascorbic acid, 2,6-dihexadicane
9	14.71	2.34	3.11	Hexadecanoic acid, ethyl ester
10	15.72	1.03	1.74	9-Octadecanoic acid, methyl ester
11	16.05	33.04	22.39	V OCtadec-9-enoic acid
12	16.20	7.83	7.89	V Octadecanoic acid
13	16.25	1.76	2.46	V Ethyl oleate
14	16.43	4.82	3.68	V Ethyl 14-Methyl-hexadecanoate
15	17.11	1.55	2.35	Tetrapentacontane 1,54-dibromate

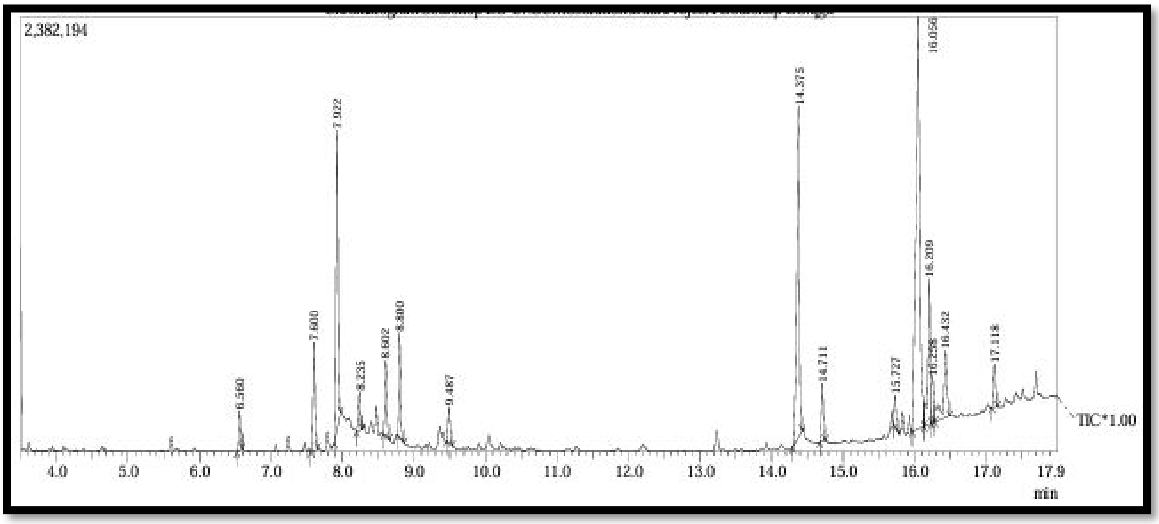

Figure 1: Chromatogram of fixed oils component of the leaves of *Annonamuricata*

Antimicrobial activity of the fixed oils against selected antibiotic-resistant microorganisms through *in vitro* assays.

The antimicrobial activities of the bacteria isolates against antibiotics are shown in Table 2. *Klebsiella* sp. and *Pseudomonas* sp. were resistant toCefuronine, Ceftazidime and Amoxil and were susceptible to Rifampicin, Azithromycin and Erythomycine, Streptomycin, Ciprofloxcin, Levofloxacin and Gentamycin while *Escherichiacoli* was resistant to all the antibiotics except Streptomycin, Ciprofloxcin, Levofloxacin and Gentamycin. *Staphylococcus*sp was susceptible to all the antibiotics.

The antibacterial susceptibility of fixed oils against on *Klebsiella* sp., *Pseudomonas* sp. and *E.coli* isolates is presented in Table 3. *E. coli* show no zone of inhibition in the different concentration fixed oil while *Klebsiella* sp.,and *Pseudomonas* sp., were susceptible and shows different zone of inhibition between the different concentrations of fixed oil used.

Table 2: Susceptibility Profile of Test Microorganisms to Selected Antibiotics.

Antibiotics	*Klebsiella* sp.	*Pseudomonas* sp.	*Escherichia* sp.	*Staphylococcus* sp.
Cefuroxime	+	+	+	-
Rifampicin	-	-	+	-
Ceftazidime	+	+	+	-
Azithromycin	-	-	+	-
Amoxil	+	+	+	-
Erythomycine	-	-	+	-
Streptomycin	-	-	-	-
Ciprofloxcin	-	-	-	-
Levofloxacin	-	-	-	-
Gentamycin	-	-	-	-

key: (+) resistant, (-) susceptible

Table 3: Antibacterial activities of the fixed oils obtained from annonamuricata leaves against *klebsiella* sp., *Pseudomonas* sp. And *Escherichia* sp.;

Concentration (μL)	Zone of inhibition (mm)		
	Klebsiella sp.	*Pseudomonas* sp.	*Escherichia* sp.
250	0	7.0	0
500	4.0	8.0	0
750	5.0	9.0	0
1000	7.0	10.0	0

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the fixed oils against the tested microorganisms.

The minimum inhibitory concentration and Minimum bactericidal concentration of *A. muricata*oil on *S. aureus* is shown in table 4. The *A. muricata*oil shows minimum inhibitory concentration of 0.5ml for *Klebsiella* sp. and 0.25ml *Pseudomonas* sp. The bactericidal concentration of*A. muricata*oil shows a bacteriostatic characteristic for all the tested organisms.

Table 4: The minimum inhibitory concentration and Minimum bactericidal concentration of fixed oil on *Klebsiella* sp. And *pseudomonas* sp.

Isolate	MIC(μL)	MBC
Klebsiella sp.	500	Bacteriostatic
Pseudomonas sp.	250	Bacteriostatic

Discussion

Plants have been used for medicinal purposes since ancient times as alternative medicine because of their diversity and flexibility of usage, availability and cost in the region, and, most importantly, the capacity to prevent unpleasant effects (Süntar, 2020; Akbar, 2020). Plants are widely used in developing nations because of their low cost, and require little technological input, making them an excellent alternative to expensive remedies (Salmerón-Manzano et al., 2020). Various parts of *Annonamuricata*(leaves, fruit, seeds, and bark) have been used to treat a range of health conditions such as infections, inflammation, diabetes, hypertension, and cancer (Zubaidi et al., 2023). The leaves have been reported to be rich in bioactive compounds like acetogenins, alkaloids, flavonoids, and essential oils, which have been shown to possess antimicrobial, antioxidant, anti-inflammatory, and anticancer properties (Errayes et al., 2020; Zubaidi et al., 2023). In traditional medicine, decoctions made from the leaves are commonly used for managing fever, pain, and parasitic infections. Modern pharmacological studies continue to support many of these traditional uses, highlighting the plant's potential as a source of natural therapeutic agents (Mutakin et al., 2022; Ilango et al., 2022).

The GC-MS analysis of fixed oils extracted from the leaves of *Annonamuricata* revealed a complex mixture of bioactive compounds, including Thymol, Caryophyllene, Caryophyllene oxide, Hexadecanoic acid ethyl ester, and Ethyl oleate, among others. These findings align with previous studies that have reported the presence of diverse secondary metabolites and essential oils in *A. muricata*, contributing to its medicinal properties (Gyesi et al., 2019; Errayes et al., 2020). Thymol, a monoterpene phenol known for its antimicrobial and antioxidant properties, was also reported in the leaf essential oils by Oladokun et al. (2021), who highlighted its role in the plant's defense mechanisms, Caryophyllene and its oxide have been reported to possess anti-inflammatory, analgesic, and antimicrobial activities (Gyrdymova&Rubtsova, 2022). The presence of fatty acid esters such as Hexadecanoic acid ethyl ester and Ethyl oleate is consistent with findings by Akompi, (2022) and Chen et al. (2023), who emphasized their contribution to the plant's lipid profile and their potential nutraceutical applications.

The antimicrobial assay of fixed oils extracted from *Annonamuricata* leaves reveals selective activity against antibiotic-resistant bacteria. The results show that E. coli exhibited complete resistance, both to conventional antibiotics and to the fixed oil, with no observable zone of inhibition, indicating a high resistance profile. *Klebsiella* sp. and *Pseudomonas* sp. showed susceptibility to the fixed oils, which manifested in variable zones of inhibition across different concentrations, suggesting dose-dependent antimicrobial effects. This selective activity supports earlier findings by Edet et al. (2022) and Silva et al. (2021), who reported that *A. muricata* extracts contain bioactive compounds such as Caryophyllene oxide and Thymol which possess antimicrobial properties, particularly against Gram-negative bacteria. The resistance of E. coli, however, may be due to the impermeable outer membrane and efficient efflux mechanisms typical of this species, as reported by MacNair& Brown, (2020);Ghai (2023).

The Minimum Inhibitory Concentration (MIC) results further support the fixed oil's antimicrobial potency, showing values of 0.5 mg/mL for Klebsiella sp. and 0.25 mg/mL for Pseudomonas sp. These relatively low MIC values indicate that the fixed oils are effective at inhibiting bacterial growth at minimal concentrations, consistent with the standards for plant-based antimicrobials. It also revealed that the oil exhibited a bacteriostatic effect rather than bactericidal activity across all tested organisms. This suggests that while the oil can inhibit microbial growth, it may not entirely eradicate the pathogens. This findings aligns with previous studies which also reported antibacteria effects of essential and fixed oils, such as those by Holanda et al. (2023) and Ibiapina et al. (2022).

Conclusion and Recommendation

The fixed oil produced by extraction of *Annonamuricata* leaf showed good antimicrobial effect against the selected bacteria with observable zones of inhibition and low values of MIC. The oils however did not have any inhibitory effect on *E. coli* and also had bacteriostatic and not bactericidal activity against all the organisms tested. The antimicrobial effects were probably caused by the chemical composition as the the GC-MS analysis shows the presence of compounds such as Thymol, Caryophyllene, and Ethyl oleate. Our findings show that *A. muricata*fixed oils can be used as a complementary agent in the management of resistant bacterial infections.Furthermore,werecommend more research should be done to isolate and purify the bioactive chemicals present in the fixed oils of *Annonamuricata* leaves in an attempt to enhance their antibacterial activity. Since the effect of bacteriostasis was reported and the effect against *E. coli* was also not effective, research should be continued to examine the mechanism of resistance and whether a combination of the oil and conventional antibiotics could have synergistic activity. Also, toxicity and pharmacokinetic tests should be conducted to ascertain the safety and therapeutic capacities of the oil in medical applications.

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