Nigerian Research Journal of Chemical Sciences (ISSN: 2682-6054)
Volume 15, Issue 1, 2026

Isolation and Characterization of (Z, Z)-9,12-Octadecadienoic Acid and Antimicrobial Efficacy of Ethyl Acetate Seed Extract of *Balanites aegyptiaca*

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Accepted: *June 6, 2026.* **Published Online***: June 11, 2026*

ABSTRACT

This study was aimed to evaluate the antimicrobial efficacy and characterize bioactive compounds in the leaves of *Balanites aegyptiaca* to determine its potential applications as remedy to some ailments. Plant materials were prepared and extracted successively using solvents in increasing order of polarity. The extracts were screened for secondary metabolites, antimicrobial and chromatographic isolation of pure bioactive compounds. The results of the study revealed the presence of alkaloids, flavonoids, tannins, saponins, fatty acids, MIC and MBC efficacy against selected pathogenic microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* spp., *Proteus* spp., and *Citrobacter* spp., (1.0, 0.5, 2.0, 1.5 and 1.5) mm and MBC values (1.5, 1.5, 2.0, 1.5 and 2.0) mm respectively. The column chromatography of the fraction F6 yielded (Z,Z)-9,12-octadecadienoic acid (linoleic acid) as confirmed by the spectroscopic analysis using FTIR, GC–MS, ^{1}H and ^{13}C NMR analyses. The result serves as a base-line data supporting the traditional medicinal use of *Balanites aegyptiaca* and identifying linoleic acid as one of its active antimicrobial constituents. Further pharmacological, toxicological investigations, purifications and elucidation of pure bioactive compounds is recommended.

Key Words: Isolation, Characterization. Antimicrobial, Efficacy, Micro-organisms

INTRODUCTION

Balanites aegyptiaca, commonly known as desert date, is a resilient tree that grows naturally in dry regions of the Middle East and Africa. Its numerous medicinal benefits have historically led to its use in folk medicine, which has sparked growing research in the fields of pharmacology

and phytochemistry. Recent research has shed light on the wide variety of bioactive substances found in this species, such as triterpenoids, alkaloids, phenolic acids, and flavonoids [1]. These substances contribute significantly to the tree's ecological resilience, offering adaptive benefits in challenging conditions, in addition to its therapeutic properties.

Recent studies have focused on the antibacterial qualities of *Balanites aegyptiaca*, demonstrating its efficacy against a range of harmful microbes. Extracts from the leaves, seeds, and bark showed significant inhibitory effects against both Gram-positive and Gram-negative bacteria, as well as some fungi, according to Umar et al. [2]. The desert date is a promising candidate for the development of natural antimicrobial drugs because of its antibacterial action, which is ascribed to the presence of certain phytochemicals that interfere with microbial cell activities.

Using advanced methods such as Gas chromatography-mass spectrometry and high-performance liquid chromatography (HPLC) to fully characterize its bioactive components, *Balanites aegyptiaca* chemical profiling has grown in popularity. Apart from simplifying the process of identifying particular constituents, these methodologies also aid in comprehending how the extracts cooperate to potentially enhance their total biological activity [3]. According to recent research, the amount of these bioactive components fluctuate throughout the plant, indicating the necessity for focused extraction techniques to optimize medicinal potential.

Extensive investigations are starting to clarify the processes by which these substances work. The study by Omer *et al.*, [4] highlights how crucial it is to comprehend how these bioactive substances interact with biological systems in order to apply them in medications and nutraceuticals. The phytochemicals' synergistic qualities could result in new treatment approaches, especially given the rise in antibiotic resistance. GC-MS analysis [5] examined the chemical profiles of aqueous extracts from the flesh and kernel, and they discovered both distinct differences and similarities between the two fruit components of *Balanites aegyptiaca* main components of both extracts were 3-O-Methyl-d-glucose, 9-Octadecenamide, and 13-Docosenamide; however, their quantities varied noticeably. The kernel has a higher concentration of saturated and unsaturated fatty acids than the meat. While 3-O-Methyl-d-glucose was present in greater amount in the meat extract, 9-Octadecenamide was the main

constituent of the kernel extract. According to the study, there is potential for using *Balanites aegyptiaca* as a source of bioactive compounds with potential medical uses, such as mood-regulating, hypolipidemic, and antioxidant qualities.

The phytochemical and proximate assessment of various parts of *Balanites aegyptiaca*, for plant's potential applications in fish sedation, anesthesia, and nutrition was conducted. Numerous bioactive substances, including alkanoids, flavonoids, tannins, saponins, terpenes, steroids, cardiac glycosides, and phenols, were discovered to be present in the root, leaf, and fruit [6]. However, the stem bark was found to have a high concentration of saponins and to be lacking in significant phytochemicals.

The antibacterial activity of extracts from the leaves and stem bark of *Balanites aegyptiaca* against clinical isolates of *Shigella dysenteriae*, *Escherichia coli*, *Staphylococcus aureus* using ethanol and ethyl acetate indicated that while both ethanol and ethyl acetate extracts have antibacterial qualities [7]. Ethyl acetate is more potent against tested microbes. However, ethanol stem bark extract had very little effect on *E. coli.* This might be due to the active components not being sufficiently degraded or concentrated. The study also demonstrates that higher extract concentrations enhance antibacterial activity, which is consistent with previous research on comparable plant extracts. The ethanol leaf extract is safe, and test animals do not have any adverse effects, according to studies on acute oral toxicity. These results indicate that extracts from *Balanites aegyptiaca* have promising potential as antimicrobial agents for treating bacterial infections, but more research is required to confirm efficacy against a wider range of pathogens and optimize their application.

Many wild edible seeds in Kashere are unexplored scientifically, despite their ethnobotanical use. In the area of novelty of this research there are few studies that have systematically analyzed wild edible plants specific to Kashere ecological zones [8]. Lack of characterization leads to underutilization and potential waste of valuable nutritional and medicinal resources. Without scientific validation, indigenous knowledge of these seeds may be lost.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The plant material (leaves) was collected from Kashere, Akko Local Government Area of Gombe State, North-Eastern Nigeria. The collected plant materials were identified and authenticated by a botanist in the Department of Biological Sciences, Federal University of Kashere, Gombe State. A voucher specimen was prepared and deposited in the departmental herbarium and voucher number: FUKB 234 was assigned for future reference.

Sample Preparation and Extraction

The sample was cleaned to remove dirt and other contaminants and dried under shade before further processing. The dried sample was pulverized to fine powder and weighed. Two hundred grams (200 g) of the powdered samples (leaves) was extracted using hot ethyl acetate in a Soxhlet extractor. The filtrate was then concentrated using rotary evaporation and stored in clean sample bottles at room temperature for further analysis [9].

Qualitative Analysis of Phytochemical Constituents

The ethyl acetate extract (2.0 g) of the leaves of *Balanites aegyptiaca* was diluted in 50 mL of distilled water and properly labeled as stock. The stock solution was gently shaken and allowed to stand for about ten minutes to ensure proper dissolution. The resulting solution was then subjected to qualitative phytochemical screening using standard procedures to determine the presence of bioactive compounds [10]

Antimicrobial Studies

The antimicrobial activity of the plant extracts was evaluated against selected pathogenic microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* spp., *Citrobacter* spp., and *Proteus* spp. These test organisms were obtained from a standard microbiology laboratory and maintained on appropriate culture media to ensure their viability and purity throughout the study.

Nutrient agar was prepared according to the manufacturer's instructions and sterilized in an autoclave at 121°C for 15 minutes. The sterilized medium was allowed to cool and then poured into sterile Petri dishes. Pure cultures of the test organisms were sub-cultured and

standardized using the 0.5 McFarland turbidity standard to ensure uniform microbial concentration.

The antimicrobial activity of the extracts was determined using the agar well diffusion method. The prepared agar plates were inoculated with the standardized microbial cultures using sterile swabs. Wells were aseptically bored into the solidified agar using a sterile cork borer, and a measured volume of each plant extract was introduced into the wells. The plates were allowed to stand briefly to permit diffusion of the extracts and were then incubated at 37 °C for 24 h.

After incubation, the antimicrobial activity was assessed by measuring the diameter of the zones of inhibition around each well in millimeters. The presence of clear zones indicated susceptibility of the microorganisms to the extracts, while larger zones indicated higher antimicrobial activity. A standard antibiotic (ciprofloxacin) was used as a positive control, while ethanol served as the negative control. The minimum inhibitory concentration (MIC) of the extracts was determined using the broth dilution method. Serial dilutions of the extracts were prepared, inoculated with the test organisms, and incubated under appropriate conditions. The lowest concentration that showed no visible growth was recorded as the MIC [11].

The minimum bactericidal concentration (MBC) of the extracts was determined following the MIC assay. Samples from the tubes that showed no visible growth during the MIC determination were sub-cultured onto freshly prepared sterile nutrient agar plates and incubated at 37°C for 24 h. The lowest concentration of the extract that showed no bacterial growth on the agar plates was recorded as the minimum bactericidal concentration

The extract that exhibited the highest antimicrobial activity was selected for further analysis. This extract was subjected to Column Chromatography for isolation and purification processes, followed by spectroscopic analysis, including Fourier Transform Infrared Spectrometry (FTIR), Gas Chromatography coupled with Mass Spectrometer (GC–MS), and Nuclear Magnetic Resonance (NMR) techniques, to identify and characterize the bioactive compounds responsible for the observed antimicrobial activity [12].

Sample for FTIR analysis was prepared by grinding 2mg sample with 100 mg KBr, mixture was pressed under high pressure to form a transparent pellet suitable for solid sample

analysis in the machine. The pellet was placed on a crystal (diamond) through which the IR beam reflects to create evanescent wave that penetrates the sample during which a minimal sample preparation was required (Agilent 5500 Compact FTIR). During the analysis, the sample was exposed to infrared radiation within the FTIR instrument. The sample absorbed infrared light at specific wavelengths corresponding to the vibrational frequencies of the chemical bonds present in the molecules. As the infrared radiation passed through the sample, the detector measured the intensity of the transmitted light over a range of wavelengths. The data obtained was processed to produce an absorption spectrum, which displayed characteristic peaks corresponding to different functional groups.

The functional groups present in the sample were identified by comparing the observed absorption peaks with standard reference data and known absorption frequencies. This enabled the identification of the chemical constituents and structural features of the sample [12].

Gas Chromatography–Mass Spectrometry Analysis

The sample was dissolved in ethanol and filtered, the filtrate was then injected into the gas chromatography (GC) system. An inert gas (helium) was used as the carrier gas to transport the sample through a capillary chromatographic column coated with a stationary phase. As the sample passed through the column, the components were separated based on their volatility and interaction with the stationary phase. Compounds with lower boiling points eluted first, while those with higher boiling points eluted later. Following separation in the GC system, the compounds entered the mass spectrometer for identification. In the MS unit, the compounds were ionized using Chemical Ionization (CI) producing charged ions. These ions were then analyzed based on their mass-to-charge ratios (m/z), generating mass spectra that provided information on the molecular weights and structural features of the compounds. The fragmentation patterns obtained from the spectra were used to deduce the structural characteristics of each compound [12].

The resulting data were analyzed to identify the compounds present in the sample by comparing the obtained mass spectra with standard reference libraries such as the NIST (National Institute of Standards and Technology) database. Quantification was carried out by

measuring the peak areas or heights in the chromatogram and comparing them with calibration curves obtained from known standards [13].

Column Chromatography

Column Parking and Elution

Glass wool was neatly placed at the tip of glass chromatography column, silica gel (60–120 mesh) was carefully packed in the column after defatting. The sample (30g) was mixed with silica to form a homogeneous mixture and placed uniformly on top of the packed column followed by fine sand to form the top layer. The column was eluted with n-hexane (100%), n-hexane: DCM (50:50), DCM (100%), DCM: EOAc (50:50), EOAc (100%), EOAc: EtOH (50:50) and EtOH (100%) [14].

Nuclear Magnetic Resonance (NMR) Analysis (^{1}H and ^{13}C)

The purified bioactive compound obtained from the most active fraction was subjected to NMR spectroscopy for detailed structural elucidation. Both proton (^{1}H NMR) and carbon-13 (^{13}C NMR) analyses were carried out to determine the molecular structure of the compound.

The sample was prepared by dissolving a known quantity of the isolated compound in a suitable deuterated solvent, such as deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (DMSO-d_6). The solution was then transferred into a clean NMR tube and placed in the NMR spectrometer (Bruker / AVANCE III HD 700 MHz NMR spectrometer) for analysis [14].

During the analysis, the sample was exposed to a strong external magnetic field, causing the nuclei of hydrogen (^{1}H) and carbon (^{13}C) atoms to align with the field. Radiofrequency radiation was then applied, resulting in resonance of the nuclei at characteristic frequencies depending on their chemical environment. The resulting signals were detected and recorded as spectra.

The ^{1}H NMR spectrum provided information on the number and types of hydrogen atoms present in the compound, as well as their chemical environments, splitting patterns, and coupling constants. Similarly, the ^{13}C NMR spectrum provided information on the carbon skeleton of the compound by indicating the number and types of carbon atoms present.

The obtained spectra were analyzed and interpreted by comparing the chemical shift values, signal multiplicity, and splitting patterns with standard reference data. This enabled the

identification and confirmation of the molecular structure of the bioactive compound isolated from the plant extract [15].

RESULTS AND DISCUSSION

The results of the various analysis are presented in Tables 1 – 7.

Table 1: Percentage Yield of Acetone and Ethyl Acetate Extracts

Sample	Acetone extract (%)	Ethyl acetate extract (%)
Leaves	8.41	4.70
Stem bark	4.25	5.38
Seeds	29.06	16.60

Table 2: Phytochemical Constituents of the Extracts

Phytochemical Constituents	F4	F5	F6
Alkaloids	+	+	+
Flavonoids	+	+	+
Tannins	+	+	–
Saponins	–	+	–
Terpenoids	+	+	+
Glycosides	+	+	+
Steroids	+	+	+
Phenols	+	+	+
Fatty acids	+	+	+

Key: F4 = Stem bark (Ethyl acetate extract), F5 = Seeds (Acetone extract), F6 = Seeds (Ethyl acetate extract)

Table 3: Antimicrobial Activity of Fractions F4–F6 against Selected Microorganisms (Zone of Inhibition in mm)

Sample	Microorganism				
	Zone of inhibition (mm)				
	E. coli	Staph.	Kleb.	Prot.	Citro.
F4	9	11	14	9	16
F5	12	7	15	11	9
F6	13	16	9	16	14
ANC	6	6	6	6	10
$C_{(30)}$	12	20	18	23	23

Key: 0.00 = No inhibition observed

Table 4: Minimum Inhibitory Concentration (MIC) of Fractions F4–F6 against Selected Microorganisms (mg/ml)

Sample	Microorganism				
	Minimum inhibitory concentration (mg/ml)				
	E. coli	Staph.	Kleb.	Prot.	Citro.
F4	2.5	4.0	2.0	3.5	4.0
F5	3.0	3.0	1.0	3.5	1.0
F6	1.0	0.5	3.0	1.0	1.5
ANC	4.0	3.5	4.0	4.0	2.5
$C_{(30)}$	2.0	0.5	0.5	0.5	0.5

Key: >5.0 = No inhibition at tested concentrations

Table 5: Minimum Bactericidal Concentration (MBC) of Fractions F1–F6 Against Selected Microorganisms (mg/ml)

Sample	Microorganism				
	Minimum bactericidal concentrations (mg/ml)				
	E.COLI	STAPH.	KLEB.	PROT.	CITRO.
F4	4.0	>5.0	3.5	5.0	>5.0
F5	4.5	4.0	2.5	4.5	2.0
F6	1.5	1.5	4.5	2.5	3.5
ANC	>5.0	4.5	>5.0	>5.0	3.5
$C_{(30)}$	4.0	1.5	1.5	1.0	1.0

Key: >5.0 = No bactericidal effect at tested concentrations

Table 6: ^{13}C NMR (Chloroform-d_1 176 MHz) Chemical Shift Data for Fraction UL_DL_3 and Comparison with Literature Values for 9,12-Octadecadienoic Acid (Z,Z)

Carbon Position	Carbon Type	Experimental δ (ppm)	Literature δ (ppm)	Carbon environment
C-1	C=O	177.13	178.2	Carboxylic acid carbon
C-2	α-CH_2	34.69	33	CH_2 adjacent to –COOH
C-3	β-CH_2	24.88	25.2	β-methylene
C-4	CH_2	27.9	28.0	Aliphatic methylene
C-5	CH_2	30.7	30.3	Aliphatic methylene
C-6	CH_2	31.0	30.7	Aliphatic methylene
C-7	CH_2	30.5	31.2	Aliphatic methylene
C-8	Allylic CH_2	31.01	32.0	CH_2 next to C=C

Carbon Position	Carbon Type	Experimental δ (ppm)	Literature δ (ppm)	Carbon environment
C-9	Olefinic CH	128.52	129.5	C=C (vinylic carbon)
C-10	Olefinic CH	130.17	131.2	C=C (vinylic carbon)
C-11	Bis-allylic CH_2	25.29	25	Between two double bonds
C-12	Olefinic CH	128.52	127	C=C (vinylic carbon)
C-13	Olefinic CH	130.17	1305	C=C (vinylic carbon)
C-14 – C-17	$(CH_2)n$	28.9 – 31.0	30.2	Aliphatic chain
C-18	CH_3	14.02	14.8	Terminal methyl

Table 7: ^1H NMR (Chloroform-d_1 700 MHz) Chemical Shift Data for Fraction UL_DL_3 and Comparison with Literature Values for 9,12-Octadecadienoic Acid (Z,Z)

Carbon Position	Carbon Type	Experimental δ (ppm)	Literature δ (ppm)	Hydrogen environment
C-1	C-OH	12.0	11.0	Carboxylic acid carbon
C-2	α-CH_2	2.53	2.23	CH_2 adjacent to –COOH
C-3	β-CH_2	1.70	1.56	β-methylene
C-4	CH_2	1.33	1.29	Aliphatic
C-5	CH_2	1.33	1.29	Aliphatic
C-6	CH_2	1.33	1.29	Aliphatic
C-7	CH_2	1.41	1.33	Aliphatic methylene chain
C-8	Allylic CH_2	2.23	1.96	CH_2 next to C=C
C-9	Olefinic CH	5.48	5.37	C=C (vinylic carbon)
C-10	Olefinic CH	5.55	5.43	C=C (vinylic carbon)
C-11	Bis-allylic CH_2	2.70	2.63	Between two double bonds
C-12	Olefinic CH	5.57	5.43	C=C (vinylic carbon)
C-13	Olefinic CH	5.55	5.37	C=C (vinylic carbon)
C-14	CH_2	2.23	1.96	Aliphatic
C-15	CH_2	1.42	1.33	Aliphatic
C-16	CH_2	1.30	1.29	Aliphatic
C-17	CH_2	1.40	1.33	Aliphatic
C-18	CH_3	1.00	0.96	Terminal methyl

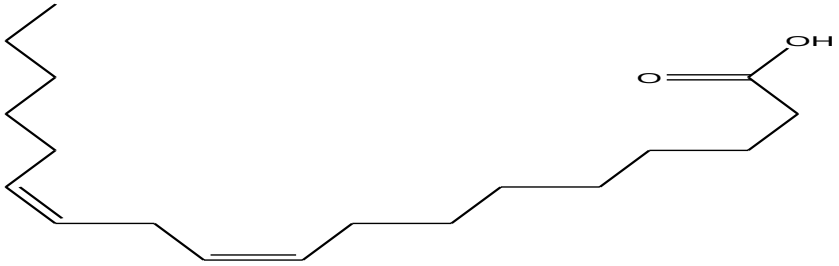

Figure 1: **(Z,Z)-9,12-OCTADECADIENOIC ACID**

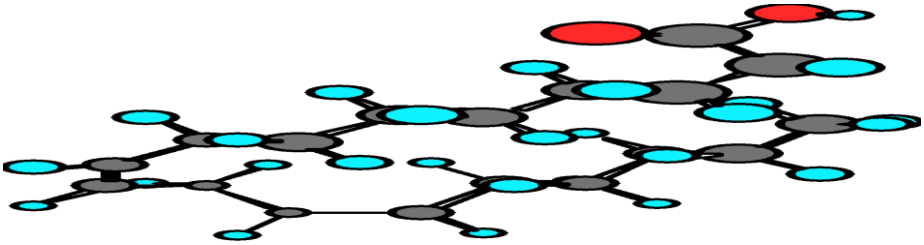

Figure 2: 3D MODEL (Z,Z)-9,12-OCTDECADIENOIC ACID

The present study evaluated the phytochemical constituents (table 2) and antimicrobial activity (table 3) of ethyl acetate *Balanites aegyptiaca* extract against a panel of bacterial pathogens, with the Minimum Inhibitory Concentration (table 4) and Minimum Bactericidal Concentration (table 5) determined using the broth micro dilution method. The extract exhibited notable antibacterial activity, with MIC and MBC values ranging from 1.0 to 2.5 mg/mL depending on the test organism. These findings contribute to the growing body of evidence supporting the therapeutic potential of *Balanites aegyptiaca* as sources of novel antimicrobial agents [16]

The results in tables 4 and 5 are particularly meaningful when contextualized within established classification criteria. According to the widely referenced [17] categorization, plant extracts with MIC and MBC values below 1.0 mg/mL are considered to possess strong antimicrobial activity, those between 1.0 and 1.5 mg/mL indicate moderate activity, and values above 2.0 mg/mL suggest weak activity. By this standard, the activity of *Balanites aegyptiaca* extract against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* spp., *Proteus* spp., and *Citrobacter* spp., (MIC = 1.0 mg/mL) can be classified as moderate, positioning it as a promising candidate for further phytochemical investigation. Comparatively, the MIC values obtained in this study are in agreement with previous reports on *Balanites aegyptiaca.* For instance, MBC values of 2.5 mg/mL [18] for wild edible plants against same bacterium, which

aligns closely with our findings was reported. However, MBC values are influenced by multiple variables including extraction solvent, plant part used, geographical origin, harvest season, and the specific microbial strain tested. The use of different methodological protocols particularly the distinction between agar diffusion and broth dilution methods can also yield substantially different results, with agar diffusion often failing to detect activity in poorly diffusible plant constituents [19].

Gram-positive bacteria were generally more susceptible to the extract than Gram-negative strains, as evidenced by the lower MIC and MBC values (tables 4&5) observed against *S. aureus* and *Proteus* spp., compared to *Escherichia coli* and *Klebsiella* spp. This differential susceptibility is consistent with the structural differences in bacterial cell envelopes. Gram-negative bacteria possess an outer membrane rich in lipopolysaccharides that acts as an effective permeability barrier, limiting the entry of hydrophobic compounds commonly found in plant extracts. The relatively higher resistance of *E. coli* (MIC = 2.0) is noteworthy, as this opportunistic pathogen is intrinsically resistant to many conventional antibiotics and represents a significant clinical challenge [20].

The mechanism underlying the antibacterial activity of *Balanites aegyptiaca* extract likely involves multiple targets, a characteristic advantage of complex phytochemical mixtures. Plant secondary metabolites such as flavonoids, alkaloids, tannins, and terpenoids, are known to exert antimicrobial effects through membrane disruption, inhibition of cell wall synthesis, interference with nucleic acid replication, and modulation of quorum sensing pathways. The synergistic action of these bioactive constituents may also explain why crude extracts often demonstrate more potent activity than isolated compounds at equivalent concentrations [21]

The MBC and MIC values reported here also carry practical significance. When MIC values approach or fall below the serum concentrations achievable through oral or topical administration, the extract warrants consideration for in vivo efficacy studies and formulation development. Furthermore, the observed activity against *Proteus* spp., and *Citrobacter* spp., is particularly encouraging given the pressing global need for new antimicrobial strategies to combat the rising threat of antibiotic resistance [22].

An observation from this study is that the MIC results correlated well with the preliminary screening data confirming the reproducibility of the antimicrobial effect. However, it should be noted that MIC determination by broth microdilution provides a quantitative endpoint that is more reliable and standardized than zone diameter measurements, making it the preferred method for comparative evaluation of plant extract potency [23].

In conclusion, the positively demonstrated MIC and MBC values established that *Balanites aegyptiaca* extract possesses antibacterial efficacy against the tested pathogens. These findings provide a scientific rationale for the traditional medicinal use of this plant and laid the groundwork for the bioassay-guided fractionation that led to identifying (Z, Z)-9,12-Octadecadienoic Acid via the ^{1}H and C-13 NMR analysis (tables 6 & 7) as an active constituents responsible for the observed antimicrobial efficacy and in agreement with Ishaku et al. [24].

CONCLUSION

The preliminary phytochemical analysis of the extract revealed the presence of flavonoids, tannins, alkaloids, saponins, terpenoids, phenolics, glycosides, and steroids. These secondary metabolites are well-documented contributors to antimicrobial activity. The MIC and MBC of *Balanites aegyptiaca* extract was determined using the broth microdilution method (CLSI guidelines, M07-A9) against a panel of bacterial pathogens. The extract exhibited dose-dependent inhibition across all test strains, with MIC values ranging from 0.5 to 1.5 mg/mL. The lowest MIC (0.125 mg/mL) was recorded against *S. aureus*, indicating the highest susceptibility. Gram-negative bacteria generally required higher extract concentrations to achieve inhibition, with *P. aeruginosa* showing the highest MIC (1.0 mg/mL). An MBC/MIC ratio ≤ 2 is considered bactericidal; a ratio > 2 indicates a bacteriostatic effect [25]. The extract demonstrated bactericidal activity against *S. aureus*, *B. subtilis*, and *S. typhi*, while exerting a bacteriostatic effect against *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. The activity of the extract was compared to ciprofloxacin/gentamicin as a positive control. The standard antibiotic yielded MIC values ranging from 0.001 to 0.008 mg/mL against the test organisms.

Recommendation

Balanites aegyptiaca seeds are known to contain steroidal saponins (balanitins), alkaloids, coumarins, triterpenes, and phenolic compounds. Authors recommend that:

1. Saponins-rich fractions should be prioritized for further bioactivity-guided chromatographic isolation of pure compounds and subsequent elucidation of the compounds.
2. Further checkerboard assay be carried out combining the seed extract with ciprofloxacin (Gram-negative), vancomycin (Gram-positive), and fluconazole (fungi) so as to calculate the fractional inhibitory concentration index (FICI $\leq$ 0.5 indicates synergy).
3. Testing of the seed extract specifically against multidrug-resistant (MDR) clinical isolates would be highly impactful.
4. MTT or resazurin assay be conducted on mammalian cell lines (e.g., HEK-293 or HepG2) to determine IC_{50} so as to calculate the selectivity index (SI = IC_{50} / MIC) — SI $\geq$ 10 is considered promising for further development.
5. The antifungal assay panel be expanded to include Candida albicans, Candida auris, Aspergillus fumigatus, and dermatophytes (Trichophyton rubrum) so as to determine the MFC (minimum fungicidal concentration) and distinguish fungistatic vs. fungicidal action.

Acknowledgement

Authors are first and foremost grateful to the management of Federal University of Kashere (FUK) for the enabling environment and moral support to carry out this research. Equally important is the funding from TETFUND Nigeria without which the entire work wouldn't have been achieved. The staff of the Department of Chemical Sciences, FUK and North-Eastern University are commended for their support.

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