UMYU Scientifica, Vol. 4 NO. 4, December 2025, Pp 197 – 214
https://doi.org/10.56919/usci.2544.017
ISSN: 2955 – 1145 (print); 2955 – 1153 (online)
*A periodical of the Faculty of Natural and Applied Sciences, UMYU, Katsina*

ORIGINAL RESEARCH ARTICLE

# Antimicrobial Properties and GC-MS Profiling of Hexane-Derived Fractions of *Moringa oleifera* Seed Obtained Using Ultrasonic Assisted Extraction Method

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## ABSTRACT
The *Moringa oleifera* seeds were sourced, identified, and extracted using ultrasonic extraction with hexane as the extraction solvent. The extract was subjected to qualitative phytochemical screening for the detection of plant metabolites using standard procedures. The isolates were collected from a tertiary hospital and were reconfirmed using standard identification procedures. The extract was fractionated and tested against the bacterial isolates; the bioactive compounds in the fractions were characterized using GC-MS. Qualitative analysis of the extract shows the presence of alkaloids, saponins, tannins, flavonoids, and phenols. Antimicrobial activity of the fractions shows broad-spectrum activity against the isolates, with mean zones of inhibition of 22 mm (butanol fraction), 22 mm (aqueous fraction), and 20.5 mm (hexane fraction). GC-MS characterization of the fractions detected key compounds like (BF oleic acid (98%), n-hexadecenoic acid (99%), Docosonoic acid (99%), Palmitoleic acid. (99%). (AF Linolaidic acid (95%), 6-octadecenoic acid (97%), cis-13- ecosenoic acid (97%), n- hexadecenoic acid (90%). (HF Phenol (87%), cis-vaccenic acid (90%), n-hexadecanoic acid (99%), 1- prophyl 9- octadecenoate heptadecanolide (95%). Conclusively, the results have revealed that the Ultrasonic Extraction method using seed and Hexane recovered phytochemicals, which significantly affected antimicrobial activity against the tested bacterial isolates.

**ARTICLE HISTORY**
Received October 07, 2025
Accepted December 22, 2025
Published December 30, 2025

**KEYWORDS**
phytochemicals, isolate, bioactive compounds



## INTRODUCTION
Medicinal plants, defined as botanical species with substances that can be used for therapeutic purposes or as precursors for the synthesis of useful drugs, represent the oldest form of healthcare known to humanity (Sofowara et al., 2013). Their use is documented across all ancient cultures, from the clay tablets of Mesopotamian civilizations and the Ebers Papyrus of ancient Egypt to the detailed compendiums of Traditional Chinese Medicine (TCM) and Ayurveda in India (Petrovska, 2012). These plants are not random weeds but sophisticated chemical entities that produce a vast array of secondary metabolites. These compounds, including alkaloids, flavonoids, terpenoids, and phenolic compounds, are primarily evolved to protect the plant against herbivores, insects, fungi, and diseases. However, in a remarkable instance of biological serendipity, these same compounds exert profound pharmacological effects on human physiology, forming the basis of their medicinal value (Croteau *et al.*, 2000). The relevance of medicinal plants in modern therapy is multifaceted, extending from traditional herbal remedies to their indispensable role in contemporary drug discovery. It is estimated that over 50% of modern clinical drugs are of natural origin, either directly isolated from plants or derived from plant-inspired synthetic compounds (Tu, 2016). Many medicinal plants are used directly, either in traditional preparations or as modern, standardized herbal extracts. Medicinal plants are far more than historical relics of pre-scientific medicine. They are a proven and invaluable reservoir of chemical diversity and complex pharmacology. From providing direct remedies in their raw form to serving as indispensable "lead compounds" in modern drug development, they remain a critical pillar of global health. As technological advances in genomics, metabolomics, and synthetic biology progress, the systematic investigation of the plant kingdom holds the promise of delivering novel therapeutics for emerging and currently untreatable diseases, ensuring that the ancient bond between plants and human health will continue to bear fruit for generations to come (Heinrich *et al.*, 2022).

*Moringa oleifera* is a plant that can grow up to 5–10 meters in a year and is planted in many countries across South Asia and West and East Africa. While it grows best in dry and sandy soil, it also tolerates poor soil in coastal areas. It is a fast-growing, drought-resistant tree native to the southern foothills of the Himalayas in northwestern India. Over the past two decades, many reports have appeared

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**How to cite**: Said, A. A., Bukar, A., & Mukhtar, M. D. (2025). Antimicrobial Properties and GC-MS Profiling of Hexane-Derived Fractions of *Moringa oleifera* Seed Obtained Using Ultrasonic Assisted Extraction Method. *UMYU Scientifica*, 4(4), 197 – 214. https://doi.org/10.56919/usci.2544.017

in mainstream scientific journals describing the nutritional and medicinal properties of *Moringa oleifera*, including its role in the treatment of malaria, intestinal worms, fungal diseases, and malnutrition, as well as its use as a water purifier. *Moringa oleifera* has been demonstrated to have antibacterial activity against some Gram-negative human pathogens, such as *Shigella shinga*, *Pseudomonas aeruginosa*, *Shigella sonnei*, and *Pseudomonas spp*, as well as some Gram-positive organisms, namely: *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus B-haemolytica*, *Bacillus subtilis*, *Sarcina lutea* and *Bacillus megaterium*. This antimicrobial effect has been attributed to different parts of the plant, such as the leaves, flowers, seeds, roots, fruit peel, and unripe pods (Torondel et al., 2014). The chloroform and ethanol extracts of *Moringa oleifera* have been found to have sanitizer/preservative activity against some foodborne microorganisms often implicated in food spoilage and foodborne illnesses (Bukar *et al.,* 2010).

*Moringa oleifera* seeds have also been demonstrated to have water-purifying properties and can be used as a substitute for chemicals in water treatment (Kawo et al., 2011). Also, the seeds and leaf extract of the plant have been shown to have antibacterial activity against bacteria isolated from patients with bacterial keratitis (Ahmad et al., 2018).

Furthermore, the *Moringa* plant has been the subject of extensive research due to its multiple uses and well-known bactericidal properties. It is rich in nutrients and, in addition to a range of industrial and medicinal applications, is used to purify water for human consumption. *Moringa* is economically important for the production of several commodities, such as oils, foods, condiments, and medicines (Viera *et al.,* 2010).

The phytochemical constituents of a plant extract are highly dependent on the solvent polarity, solvent-to-plant material ratio, particle size of the plant material, temperature, and extraction method. Several studies reported that the nature of solvents and extraction methods are the two important factors affecting the phytochemical composition of plant extracts. In recent years, extraction methods, namely Ultrasonic Assisted Extraction (UAE) and Microwave Assisted Extraction (MAE), have been considered green extraction methods and have been extensively studied for the extraction of phytochemicals from various plant materials. The results of previous studies indicated that MAE and UAE are efficient methods for extraction of phytochemicals from plant materials (Kannamba *et al.,* 2017).

Ultrasound-assisted extraction (UAE) has been applied at industrial and laboratory scales in recent years to extract bioactive compounds from plants. Ultrasound frequencies ranging from 20 kHz to 2000 kHz are used in UAE. The UAE has extracted a wide range of bioactive compounds using various solvents. According to a study by Rodríguez-Pérez, the UAE technique produced higher phenolic content than the maceration technique when crude *Moringa oleifera* extracts were extracted using 25 mL of solvent for 15 minutes at room temperature (Rodríguez-Pérez *et al.,* 2015). Hexane's low viscosity and high vapor pressure facilitate efficient cavitation during UAE, improving mass transfer and compound yield. A Study by Rodríguez-Pérez et al. (2015) showed that UAE with non-polar solvents, such as hexane, increases the extraction efficiency of lipophilic phytochemicals while reducing time and solvent volume. UAE operates at lower temperatures than Soxhlet or maceration, thereby preventing the degradation of heat-sensitive antimicrobial compounds. Hexane's low boiling point (69°C) further supports gentle extraction.

Moreover, Lin *et al.* (2021) showed that higher flavonoid concentrations (47.04 mg QE/g MOLs dried weight) were obtained when 52% hexane was used as the solvent. Like MAE, the UAE approach has been successful in obtaining higher levels of flavonoid and phenolic content while using less solvent and a shorter extraction period (Ivanovs and Blumberga, 2017). Wen *et al.* (2018) also examined recent applications of ultrasonics in business and provided evidence of the technique's substantial economic potential. In both small and large-scale applications, ultrasound-assisted extraction seems to be inexpensive. (Medina-Torres *et al.,* 2017).

The selection of hexane as the primary extraction solvent in this study, particularly when combined with ultrasonic-assisted extraction (UAE), is strategically justified by several scientific and practical considerations aligned with the research objectives.

Extraction is a critical step in the discovery of bioactive constituents from plant materials. The most commonly used extraction techniques include conventional methods such as maceration, percolation, infusion, decoction, and hot continuous extraction (Newman and Craggman 2020). Recently, alternative methods such as ultrasound-assisted solvent extraction (UASE) and microwave-assisted solvent extraction (MASE) have gained increasing popularity over the last three decades. The yields and bioactivities of extracts prepared using different extraction methods have been reported to vary. Extracts from different parts of *Moringa oleifera* obtained using various extraction methods have been shown to possess antibacterial properties. Many studies test only one or two extraction methods or solvents. There is a lack of systematic comparison across conventional and advanced techniques. Few studies directly correlate specific phytochemicals recovered by each extraction method with antimicrobial efficacy. Different researchers have reported works on different parts of *Moringa oleifera* plant using traditional extractions methods without comparing the methods of extraction (maceration, percolation, soxhlet) (Bukar *et al.* 2010; Lawan *et al.* 2020; Ahmad *et al.*2018;), but were not able to establish the best extraction methods (previous and recently used techniques) that gives the best phytochemical composition that would translate to good antimicrobial activity.

## MATERIALS AND METHODS

### Plant material

The *Moringa oleifera* plants were collected from a farm at Rafin Gusa, Kaduna State, and identified. The seed were washed thoroughly to remove adhering material and dried at room temperature. The seeds were further ground by means of an electric blender into fine powder (Mukhtar and Bukar, 2008)

### Preparation of plant extracts

100 g of powder from different parts of the plant was immersed in 500 mL of the solvents, separately, in beakers. Extraction was carried out by placing the ultrasonic machine inside a beaker containing the plant extract and solvent. The ultrasound-assisted extractor was operated for 2 hours, with 20-minute intervals (Kannamba *et al.,* 2017).

The extract was concentrated by rotary vacuum evaporator and then further dried in a water bath to remove residual solvents. Physiochemical features of the extract observed included percentage recovery, pH, solubility, texture, colour, odour, taste, turbidity, consistency, and particle size and state (solid, liquid, or gas).

### Phytochemical Analysis

The extract was analyzed for the presence of alkaloids, Saponins, tannins, Flavonoids, and Steroids using a standard method (Kumar *et al*., 2010).

### Collection of Clinical Isolates

*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* were collected from Saint Gerard Catholic Hospital, Kaduna. Identification of these isolates was reconfirmed using Gram staining and biochemical tests.

### Identification of Test Organisms

### Identification of *Staphylococcus aureus*

### Catalase Test

Two millilitres of the hydrogen peroxide solution were poured into a test tube. Using a glass rod, several colonies of the test organism were removed and immersed in the hydrogen peroxide solution. Immediate bubbling was observed. The presence of bubbles indicated a positive for *Staphylococcus* species (Cheesbrough, 2002).

### *Coagulase Test*

Two separate drops of distilled water were placed on a glass slide. A colony of the test organism (previously checked by Gram staining) was emulsified in each drop to produce two thick suspensions. A loopful of plasma was added to one of the suspensions, and the suspension was mixed gently. Clumping of the organisms was observed within 10 seconds. No plasma was added to the second suspension; this was used to differentiate any granular appearance of the organism from true coagulase clumping. Positive coagulase reaction indicated *Staphylococcus aureus* (Cheesbrough, 2002).

### Identification of Streptococcus pneumoniae

### Optochin Sensitivity.

Optochin (p) disk (6mm, 5 microgramme) SIGMA-ALDRICH 74042 was obtained from commercial vendors. Using an inoculating loop, two suspect colonies of a pure culture to be tested were streaked on blood agar. An optochin disk was placed within the streaked area of the plate. The blood agar plate was incubated at 37 $^0$C with carbon dioxide (candle jar) for 24 hours. The growth on the blood agar plate was observed near the optochin disk. The absence of growth indicated sensitivity to optochin, confirming S*treptococcus pneumoniae* (Cheesbrough, 2002).

### Identification *Pseudomonas aeruginosa*

### Kliegler Iron Agar (KIA)

*Pseudomonas aeruginosa* produces an alkaline/alkaline (red slant/red butt) reaction with no production of $H_2S$ on KIA. There is usually no gas production, but the medium may show signs of cracking or bleaching due to $CO_2$ produced by the pyocyanin pigment. KIA is a differential medium used primarily to distinguish between members of the *Enterobacteriaceae* family based on their fermentation patterns. *P. aeruginosa*, being a non-fermenter, gives a very characteristic and distinct reaction.

KIA contains two sugars: Lactose (1%), which is present at a higher concentration, primarily in the slant. Glucose (0.1%), present at a lower concentration, primarily in the butt. It also contains peptone, a pH indicator (phenol red), and ferrous sulfate for detecting $H_2S$. *P. aeruginosa* does not ferment glucose anaerobically in the sealed butt of the tube. It is an oxidative organism. It may oxidize the small amount of glucose initially, producing a slight amount of acid. However, it quickly depletes the glucose and then reverts to oxidizing peptones (protein) as an energy source. Peptone oxidation produces alkaline amines, which raise the pH. The butt turns red (alkaline). *P. aeruginosa* does not ferment lactose. Without acid production from lactose, the slant remains alkaline due to the oxidative metabolism of peptones. The slant also turns red (alkaline). *P. aeruginosa* does not produce hydrogen sulfide, so there is no blackening of the medium. Typically, no gas is produced from fermentation. However, you may sometimes see the agar crack or lift. This is not from fermentative gas but from $CO_2$ produced during oxidative metabolism. The medium may exhibit a greenish-blue discoloration or a "bleached" appearance

due to the diffusion of the characteristic pyocyanin and pyoverdine pigments produced by the bacterium.

The KIA (SIGMA ALDRICH) prepared in test tubes was inoculated with the test organism using a straight wire: first stabbing the butt, then streaking the slope in a zig–zag pattern, followed by incubation at 37 °C for 24 hours. After incubation, the test tubes were observed for the expected reactions (Bukar *et al.*,2009).

**Oxidase Test**

A piece of filter paper was placed in a clean Petri dish, and 2 drops of freshly prepared oxidase reagent were added. Using a glass rod, a colony of the test organism was removed and smeared onto the filter paper. A purple colour developed within a few seconds (Cheesbrough, 2002).

**Identification of Escherichia coli**

**Indole Test**

Peptone was prepared according to manufacturer's instructions. The growth obtained was inoculated into peptone water and incubated for 24 hours at 37 $^0$C. After 24 hours, 0.3 ml of Kovacs reagent was added to check for a positive or negative result; the appearance of a red colour indicated indole-positive, confirming *Escherichia coli* (Chessebrough, 2006).

**Citrate Test**

About 2.4g of citrate agar was dissolved in 100ml of distilled water. About 10ml of citrate medium was dispensed into each tube, then the tubes were covered, sterilized, and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms onto the agar surface. The lack of change from green to blue indicates no utilization of citrate, which confirms the presence of *Escherichia coli* (Cheesebrough, 2006).

**Methyl Red (MP) and Voges proskauer (VP) Test**

The test organism was cultured in peptone-glucose water in a test tube and incubated at 37 °C for 72 hours. After incubation, 2ml of the broth culture was transferred to a sterile test tube, 2 drops of methyl red indicator were added, and the mixture was shaken vigorously to mix and allowed to stand for a few minutes. The formation of a bright red colour indicates the presence of *Escherichia coli* (Oyeleke and Manga, 2008).

**Identification of Isolate enteric bacteria using API20E system**

The isolates collected were further confirmed using Analytical Profile index 20E, a standardized identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods (George *et al.,* 2018). The API 20E strips consist of 20 microtubes containing dehydrated substrates to detect enzymatic activity or the fermentation of sugars by the inoculated organisms.

**Bioassay**

**Standardization of bacterial inoculum**

The inoculum size of all bacterial isolates tested was standardized using overnight broth cultures prepared by inoculating 2 loopfuls of well-isolated colonies of the test bacteria into 10 ml of broth, which was then incubated at 37 °c for 24 hours. A loopful of the overnight broth culture was diluted in 4 ml of sterile physiological saline (0.8% w/v) until its turbidity matched that of the 0.5 MacFarland Standard (Cheesebrough, 2002). This corresponded to $1.5\times10^8$ CFU/ml.

**Solvent partitioning of the extract of Moringa Oleifera.**

One hundred grams of the extract were dissolved separately in 250 mL of sterile distilled water in a separatory funnel and partitioned using organic solvents in order of polarity. The solvents used were n-hexane, chloroform, ethanol, and butanol. Starting with n-hexane, 200 ml was added to the mixture and gently swirled. The mixture was left to settle into layers before the n-hexane fraction was collected. This process was repeated until no further change in the colour of the n-hexane occurred. The n-hexane fraction was concentrated, dried, and stored in a fridge for further use. The resulting aqueous phase was reconcentrated to remove traces of n-hexane. The residue was then further extracted with 200 ml of chloroform. The chloroform fraction was similarly concentrated, dried, and stored in a fridge for later use. The ethanol and butanol fractions were collected using the same procedure. The remaining aqueous fraction was concentrated, dried, and kept in a fridge for future use (Alayande *et al.*, 2017).

**Preparation of Extract Concentration.**

Concentrations of 25mg/ml, 12.5mg/ml, 6.25mg/ml, and 3.125mg/ml were prepared by dissolving 50g of the extract in 2ml of DMSO. Serial doubling dilutions were carried out by adding 1 mL of DMSO at each dilution (Bukar *et al.,* 2010).

**Antimicrobial Assay**

The antimicrobial activity of the extract was determined using the agar well diffusion method. Plates of Mueller-Hinton agar were dried in an oven to remove excess surface moisture. Standardized microbial inoculum was evenly distributed using a sterile swab stick. Wells of 5mm in diameter were punched into the agar with a stainless-steel borer and filled with different concentrations of the plant extracts. Control wells containing antibiotics were also included. The plates were incubated for 24 hours at 37°c. The antimicrobial activity was assessed by measuring the zone of inhibition diameter after 24 hours.

**Minimum Inhibitory Concentration (MIC)**

The Minimum Inhibitory Concentration (MIC) of the active plant extract was determined using the tube dilution method. Test concentrations were prepared and added to sterile capped test tubes to cover the chosen dilution range in duplicate, each containing an equal volume of Mueller-Hinton broth (2 ml). A 0.1 ml of standardized bacterial inoculum was then added. After overnight incubation at 37 °c, the lowest concentration of the extract at which no turbidity was observed was recorded as the Minimum Inhibitory Concentration.

**Minimum Bactericidal Concentration**

Sterile Mueller-Hinton agar plates were inoculated with samples from the MIC tubes that showed no visible bacterial or fungal growth. The lowest concentration at which no growth occurred on the medium was considered the Minimum Bacteriocidal Concentration.

**Determination of Bioactive Compounds in Fractions.**

The Fractions were cleaned by passing them through an anhydrous sodium sulphate phase and reconstituted in 2-propanol (HPLC grade). The extracts were concentrated by solvent evaporation in a fume hood to about 2.0 mL. The final extracts were filtered through 0.45 µm PTFE membrane (Joseph *et al.,* 2023).

**GC-MS Analysis of the extract**

To prepare the samples for analysis, the target compounds (analytes) were first isolated. This was achieved using liquid-liquid extraction, which partitions analytes between two unmixable liquids, and solid-phase extraction, which uses a cartridge to selectively bind and release them. For volatile compounds, a technique was used in which a coated fibre absorbed analytes from the vapour in the sample vial; these analytes were later released by heating in the instrument. Some non-volatile compounds were chemically modified to make them suitable for analysis. The final sample extract was dissolved in a volatile solvent to a precise volume. For sample introduction, a small, precise volume of this solution was injected into a heated injector, which instantly vaporized it. A specific injector was used that could control the temperature, which is excellent for handling samples with a wide range of volatilities or for protecting sensitive compounds from degradation. The separation was performed using gas chromatography (GC). A hydrogen gas stream carried the vaporized sample through a long, narrow column housed inside a temperature-controlled oven. As the oven temperature increased, the different compounds in the sample interacted with the column's coating to varying degrees, leading to their separation and exit from the column at different times.

After separation, the compounds entered a mass spectrometer. Here, they were bombarded with high-energy electrons, which broke them into charged fragments. These fragment ions were then separated based on their mass. The most common instruments used either an electrical field to filter masses or a trap to hold and then eject them by mass. Finally, a computer system processed all the data. The primary output was a graph called a Total Ion Chromatogram (TIC), where each peak represents a separated compound. For each peak, a mass spectrum was recorded, which serves as a unique fingerprint. This spectrum was compared against a large reference library to help identify the unknown compound. To determine the concentration, the size of the peak was compared to a calibration curve created from standards of known concentration described by the National Institute of Standards and Technology (NIST) library).

## RESULTS

**Physical characteristics of the plant extract.**

Table 1 shows the physical characteristics of the extract. The ultrasonic-assisted extraction of *Moringa oleifera* seeds with hexane yielded 41% (41.3 g) of a cream-coloured, oily extract from 100 g of starting material. This high extraction efficiency underscores the suitability of hexane as a solvent for effectively recovering lipophilic compounds from the oil-rich seed matrix. The physical characteristics of the extract—specifically its creamy hue and oily consistency—are consistent with a concentrate rich in non-polar constituents such as fixed oils and fatty acids, which were subsequently characterized for their antimicrobial potential.

**Table 1: Physical Characteristics of the Extract.**

| Parameters | HSU |
|---|---|
| Weight powder (g) | 100 |
| Weight extract (g) | 41.3 |
| % Recover extract | 41 |
| Color of extract | Cream |
| Texture | Oily |

**Key: HSU= hexane seed ultrasonic.**

**Table 2: Phytochemical Contents of the extract *Moringa* Seed,**

| Phytochemicals | HSU |
|---|---|
| Alkaloids | + |
| Saponin | + |
| Tannins | + |
| Flavonoids | + |
| Steroids | - |
| Phenols | + |

**Key: HSU hexane seeds ultrasonic**

Table 2 shows the Phytochemical contents of the extract. The qualitative phytochemical screening of the hexane seed extract (HSU) revealed the presence of a broad spectrum of secondary metabolites. The extract tested positive for alkaloids, saponins, tannins, flavonoids, and phenols, indicating a rich composition of potentially bioactive compounds with diverse pharmacological properties, including antimicrobial, antioxidant, and anti-inflammatory activities. Notably, the extract tested

negative for steroids. The concurrent presence of these phytoconstituents, particularly flavonoids and phenols, provides a preliminary chemical basis for the observed antimicrobial efficacy of the extract and aligns with the known bioactivity profile of *Moringa oleifera* seeds.

**Antibacterial Activity of Fractions of the Most Active Extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus.***

Table 3 presents the mean antimicrobial activity (± standard deviation) of different solvent fractions from the extract of *Moringa oleifera* against two Gram-negative and Gram-positive bacterial strains, specifically *Pseudomonas aeruginosa* (clinical isolate and ATCC 27833) and *Staphylococcus aureus* (clinical isolate and ATCC 25923). The results are expressed in mg/ml, with statistical significance determined at $P < 0.05$ using Pitcher's LSD. Values with different superscripts in each column indicate significant differences. The bioactivity-guided fractionation revealed distinct antimicrobial profiles among the solvent-partitioned fractions against both clinical and reference strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The butanol fraction (BF) exhibited the most potent and broad-spectrum activity, particularly against *S. aureus*, with inhibition zones comparable to or exceeding standard antibiotics like streptomycin (STR) and ampicillin (AMP). Notably, the aqueous fraction (AF) showed strong efficacy against the clinical isolate of *P. aeruginosa* but markedly reduced activity against its ATCC counterpart, suggesting strain-specific susceptibility. While the hexane (HF) and chloroform (CF) fractions demonstrated significant activity, the ethyl acetate fraction (EF) was consistently the least effective, highlighting that the antimicrobial principles were concentrated in the more polar butanol and aqueous fractions, which contained a synergistic blend of bioactive compounds.

**Table 3 Antibacterial activity (Mean ±Standard Deviation) of the fractions of the extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus***

| Fractions(25 mg/ml) | *Pseudomonas aeruginosa* (mm) | *Pseudomonas aeruginosa* (ATCC 27833) | *Staphylococcus aureus (mm)* | *Staphylococcus aureus* (ATCC 25923) |
|---|---|---|---|---|
| HF | 22 ±1.63a | 18 ± 0.28b | 22 ± 1.90ab | 15 ± 0.94cde |
| CF | 22 ± 1.70a | 21 ± 1.28b | 17 ± 0.32cde | 12 ± 1.63e |
| EF | 22 ± 0.70a | 19 ± 0.00b | 13 ± 0.02e | 11 ± 1.63e |
| BF | 16 ± 0.91b | 22 ± 0.82a | 25 ± 0.30a | 22 ± 0.63ab |
| AF | 23 ± 1.90a | 9.00 ± 1.40c | 22 ± 0.5ab | 19 ± 1.87bcd |
| Control (Antibiotics) | | | | |
| CIP | 22 ± 0.30ab | 24 ±1.62a | 26 ± 0.63a | 24 ± 1.72a |
| STR | 16 ±0.63b | 23 ± 0.40a | 20 ± 0.00bcd | 23 ±0.82ab |
| CTR | 25 ±0.51a | 26 ± 0.91a | 21 ±1.73abc | 20 ± 1.40abc |
| AMP | 17 ±0.00b | 24 ± 1.47a | 16 .82de | 14 ± 0.00de |

**Means along column with different superscripts for each factor are significantly different at P<0.05 using Pitcher's LSD**

**CIP= Ciprofloxacin, STP= Streptomycin, CTR= Ceftriaxone, AMP= Ampicillin, HF= Hexane fraction, CF= Chloroform Fraction, EF= Ethyl acetate Fraction, BF= Butanol Fraction. AF= Aqueous Fraction.**

**Table 4. Antibacterial activity (Mean ±Standard Deviation) of the fractions of the extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus***

| Fractions | *E. coli* (mm) | *E. coli* (ATCC 25922) | *Streptococcus Pneumoniae (mm)* | *Streptococcus Pneumoniae* (ATCC 49619) |
|---|---|---|---|---|
| HF | 22 ± 1.36ab | 23 ± 0.20a | 18 ± 0.78b | 12 ± 0.00c |
| CF | 16 ±0.80c | 12 ± 0.00c | 14 ±0.28c | 10 ± 1.03c |
| EF | 15 ± 0.82c | 18 ± 1.70b | 11 ± 0.82c | 9 ± 0.40c |
| BF | 23 ± 1.73a | 18 ± 0.82b | 18 ± 0.10b | 16 ± 1.94b |
| AF | 22 ± 0.82ab | 16 ± 1.30bc | 11 ± 0.02c | 9.00± 0.00c |
| Control (Antibiotics) | | | | |
| CIP | 16 ± 0.94c | 13 ± 1.28c | 19 ± 0.80b | 18 ± 0.28a |
| STR | 16 ± 0.00c | 13 ± 0.32c | 21 ± 0.40b | 18 ± 0.71a |
| CTR | 17 ± 1.90bc | 13 ± 0.91c | 20 ± 0.00b | 20 ± 0.63a |
| AMP | 26 ± 0.20a | 20 ±1.20ab | 26 ± 0.78a | 18 ± 0.00a |

**Means along columns with different superscripts for each factor are significantly different at P<0.05 using Pitcher's LSD**

**CIP= Ciprofloxacin, STP= Streptomycin, CTR= Ceftriaxone, AMP= Ampicillin, HF= Hexane fraction, CF= Chloroform Fraction, EF= Ethyl acetate Fraction, BF= Butanol Fraction. AF= Aqueous Fraction.**

**Table 5 Minimum Inhibitory Concentrations of the fractions in (mg/ml)**

| Fractions (mg/mL) | *Pseudomonas aeruginosa* | *Pseudomonas aeruginosa* (ATCC 27833) | *Staphylococcus aureus* | *Staphylococcus aureus* (ATCC 25923) |
|---|---|---|---|---|
| HF | 5 | 10 | 5 | 10 |
| CF | 5 | 5 | 10 | 10 |
| EF | 10 | 10 | 10 | 10 |
| BF | 5 | 5 | 2.5 | 5 |
| AF | 5 | 10 | 5 | 10 |

**HF= Hexane fraction, CF= Chloroform Fraction, EF= Ethyl acetate Fraction, MF= Butanol Fraction. AF= Aqueous Fraction**

**Table 6 Minimum Bacteriocidal Concentrations of the Fractions (mg/ml)**

| Fractions (mg/mL) | *Pseudomonas aeruginosa* | *Pseudomonas aeruginosa* (ATCC 27833) | *Staphylococcus aureus* | *Staphylococcus aureus* (ATCC 25923) |
|---|---|---|---|---|
| HF | 10 | >10 | 10 | >10 |
| CF | 10 | 10 | >10 | >10 |
| EF | >10 | >10 | >10 | >10 |
| BF | 10 | 10 | 5 | 10 |
| AF | 10 | >10 | 10 | >10 |

**HF= Hexane fraction, CF= Chloroform Fraction, EF= Ethyl acetate Fraction, MF= Butanol Fraction. AF= Aqueous Fraction**

**Table 7 Minimum Inhibitory Concentrations of the fractions (mg/ml)**

| Fractions (mg/mL) | *E. coli* | *E. coli* (ATCC 25922) | *Streptococcus Pneumoniae* | *Streptococcus Pneumoniae* (ATCC 49619) |
|---|---|---|---|---|
| HF | 5 | 5 | 10 | 10 |
| CF | 10 | 10 | 10 | 10 |
| EF | 10 | 10 | 10 | 10 |
| BF | 5 | 10 | 10 | 10 |
| AF | 5 | 10 | 10 | 10 |

**HF= Hexane fraction, CF= Chloroform Fraction, EF= Ethyl acetate Fraction, MF= Butanol Fraction. AF= Aqueous Fraction**

**Table 8 Minimum Bacteriocidal Concentrations of the Fractions (mg/ml)**

| Fractions (mg/mL) | *E. coli* | *E. coli* (ATCC 25922) | *Streptococcus Pneumoniae* | *Streptococcus Pneumoniae* (ATCC 49619) |
|---|---|---|---|---|
| HF | 10 | 10 | >10 | >10 |
| CF | >10 | >10 | >10 | >10 |
| EF | >10 | >10 | >10 | >10 |
| BF | 10 | >10 | >10 | >10 |
| AF | 10 | >10 | >10 | >10 |

**HF= Hexane fraction, CF= Chloroform Fraction, EF= Ethyl acetate Fraction, MF= Butanol Fraction. AF= Aqueous Fraction**

**Antibacterial Activity of Fractions of the Most Active Extract against *Escherichia coli* and *Streptococcus pneumoniae***

Table 4 shows the antibacterial activity (expressed as Mean ± Standard Deviation in mg/ml) of various solvent fractions derived from the extract of *Moringa oleifera* seed against clinical and ATCC strains *of Escherichia coli* and *Streptococcus pneumoniae*. The effectiveness of the fractions was compared with that of standard antibiotics. Different superscripts indicate significant differences ($P < 0.05$) among treatments. The antimicrobial evaluation of the fractions against *Escherichia coli* and *Streptococcus pneumoniae* demonstrated variable efficacy, with the hexane (HF) and butanol (BF) fractions exhibiting the strongest inhibitory activity. Against *E. coli*, HF and BF produced inhibition zones (22-23 mm) that were significantly larger than those of several standard antibiotics, including ciprofloxacin, streptomycin, and ceftriaxone, although they were surpassed by ampicillin. In contrast, all fractions displayed moderate to low activity against *S. pneumoniae*, with BF showing the highest effect (16-18 mm), which remained inferior to the potent inhibition by standard antibiotics. The chloroform (CF), ethyl acetate (EF), and aqueous (AF) fractions showed consistently weaker activity across both pathogens, reinforcing that the primary antibacterial constituents against these Gram-negative and Gram-positive bacteria were concentrated in the intermediate-polarity HF and BF fractions.

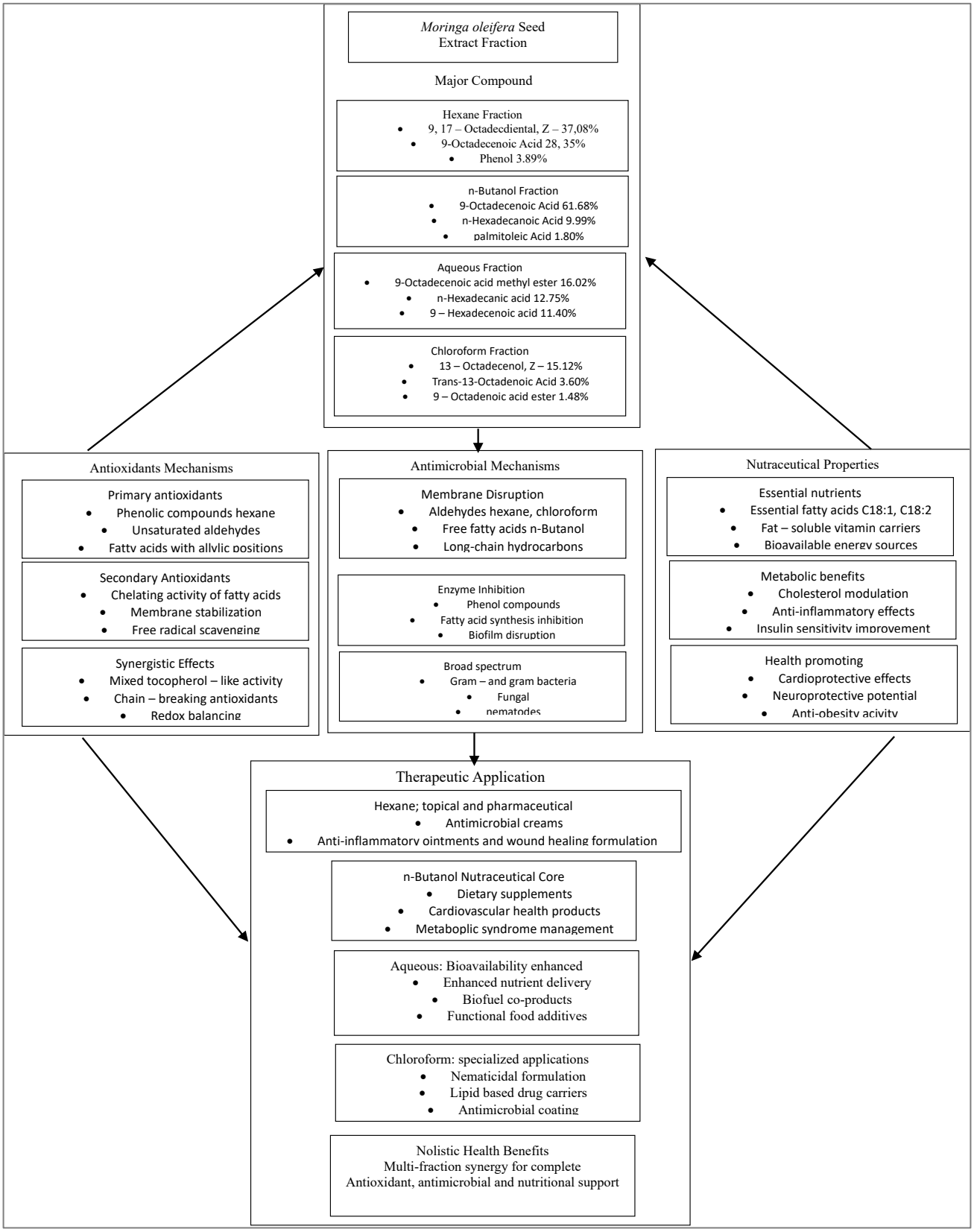


**Figure 1: Summary of the GC-MS Analysis**

**Minimum Inhibitory Concentration and Minimum Bacteriocidal Concentration of the fractions.**

Tables 5 and 6 present the Minimum Inhibitory Concentrations of the Fractions. HF, CF, BF, and AF showed low MICs against the clinical isolate (5 mg/ml), with BF and CF being equally effective against the ATCC strain (5 mg/ml). EF showed the lowest MIC against both strains (10 mg/mL). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays quantified the potency and cidal action of the fractions, revealing that the butanol fraction (BF) was the most effective. BF displayed the lowest overall MIC values, including a notably potent MIC

of 2.5 mg/mL against the clinical isolate of *Staphylococcus aureus*, and achieved bactericidal activity (MBC = 5 mg/mL) against the same strain, indicating its ability not only to inhibit but to kill this pathogen at a low concentration. In contrast, the ethyl acetate fraction (EF) consistently showed the highest MIC and MBC values (≥10 mg/mL), reflecting its weak activity, while the hexane (HF), chloroform (CF), and aqueous (AF) fractions exhibited intermediate and predominantly bacteriostatic effects, with MBC values often exceeding the highest tested concentration (>10 mg/mL) against several strains.

**Table 9 Bioactive Compounds in n-Butanol Fraction**

| Peak No | Compounds | RT (min) | Molecular Formula | Molecular Weight (g/mol) | Peak Area % |
|---|---|---|---|---|---|
| 1 | Octanal (86%) | 3.55 | $C_8H_{16}O$ | 128 | 0.65 |
| 2 | Octenal, (E) – (83%) | 4.254 | $C_8H_{14}O$ | 126 | 0.32 |
| 3 | Nonanal (78%) | 4.832 | $C_9H_{18}O$ | 142 | 1.13 |
| 4 | 2- Nonenal, (E) (86%) | 5.559 | $C_9H_{16}O$ | 140 | 0.35 |
| 5 | 4- Decenal, (E) (52%) | 6.017 | $C_{10}H_{18}O$ | 154 | 0.47 |
| 6 | 2-Decenal (91%) | 6.858 | $C_{10}H_{18}O$ | 154 | 3.25 |
| 7 | Nonanoic acid (90%) | 7.035 | $C_9H_{18}O_2$ | 158 | 0.36 |
| 8 | Cis- Undec-4-enal (64%) | 7.241 | $C_{11}H_{20}O$ | 168 | 0.66 |
| 9 | (E) – Tetradec- 2-enal (86%) or (E) - Hexadec – 2- enal (86) | 8.054 | $C_{14}H_{26}O$<br>$C_{16}H_{30}O$ | 210<br>238 | 3.38 |
| 10 | 8-Heptadecene (94%) | 11.229 | $C_{17}H_{34}$ | 238 | 0.59 |
| 11 | Palmitoleic acid (99%) | 13.667 | $C_{16}H_{30}O_2$ | 254 | 1.80 |
| 12 | n- Hexadecanoic acid (99%) | 13.930 | $C_{16}H_{32}O_2$ | 256 | 9.99 |
| 13 | 9- Octadecenoic acid (Z) – methy ester (99%) | 14.852 | $C_{19}H_{36}O_2$ | 296 | 1.04 |
| 14 | 9- Octadecenoic acid (99%) or 6 – Octadecenoic acid (Z) – (99%) | 15.572 | $C_{18}H_{34}O_2$<br>$C_{18}H_{34}O_2$ | 282<br>282 | 61.68 |
| 15 | 9,12- Octadecadienoic acid (Z, Z)- (98%) | 15.990 | $C_{18}H_{32}O_2$ | 280 | 0.69 |
| 16 | Oleic acid (98%) | 16.082 | $C_{18}H_{34}O_2$ | 282 | 0.64 |
| 17 | 9- Octadecenoic acid, (E) (99%) | 16.265 | $C_{18}H_{34}O_2$ | 282 | 0.34 |
| 18 | Oleic acid (98%) | 16.431 | $C_{18}H_{34}O_2$ | 282 | 0.76 |
| 19 | Octadecenioc acid (E)- (90%) | 16.614 | $C_{18}H_{34}O_2$ | 282 | 0.35 |
| 20 | 9- Octadecenoic acid, (E)- (96%) | 16.787 | $C_{18}H_{34}O_2$ | 282 | 0.93 |
| 21 | Cis -11- Eicosenoic acid (99%) | 17.049 | $C_{20}H_{38}O_2$ | 310 | 2.62 |
| 22 | Eicosanoic acid (99%) | 17.341 | $C_{20}H_{40}O_2$ | 313 | 2.82 |
| 23 | 13- Octadecenal, (Z)- (87%) | 17.667 | $C_{18}H_{34}O$ | 266 | 0.75 |
| 24 | 9,17- Octadecadienal, (Z) (91%) | 17.964 | $C_{18}H_{32}O$ | 264 | 0.70 |
| 25 | Cis- Vaccenic acid (89%) | 18.605 | $C_{18}H_{34}O_2$ | 282 | 0.36 |
| 26 | 1,2- Benisothiazole, 3-(hexahydro-1H-azepin-1-yl)- 1,1-dioxide (90%) | 18.914 | $C_{13}H_{16}N_2O2_S$ | 272 | 0.32 |
| 27 | 1-Hydroxycyclododecanecarbonitrile (64%) | 19.229 | $C_{13}H_{23}NO$ | 209 | 0.42 |
| 28 | Octadecanoic acid, 11- methyle-, methyl ester (90%)<br>Or<br>Docosanoic acid, methyl ester (90%) | 19.469 | $C_{20}H_{40}O_2$<br>$C_{23}H_{46}O_2$ | 299<br>355 | 0.33 |
| 29 | Docosanioc acid (99%) | 20.328 | $C_{22}H_{44}O_2$ | 340 | 2.11 |

For Isomers (peaks 9, 13, 14, 16… 20), the MW is the same, but the retention time differs.
Methyl esters (peak 13,28) have a higher MW due to the -OCH3 group.

Tables 7 and 8 present that, for *E. coli*, fractions HF, BF, and AF demonstrated low MICs against the clinical isolate (5 mg/ml). HF also showed a Low MIC against the ATCC strain at 5 mg/mL. CF and EF showed reduced potency with MICs of 10 mg/mL. In terms of bactericidal action, HF, BF, and AF were bactericidal against the clinical *E. coli* at 10 mg/ml. However, only HF was bactericidal against the *E. coli* ATCC strain at 10 mg/ml, with BF and AF only inhibitory at that concentration (>10 mg/ml). CF and EF were generally ineffective as bactericides against *E. coli* at the tested concentrations.

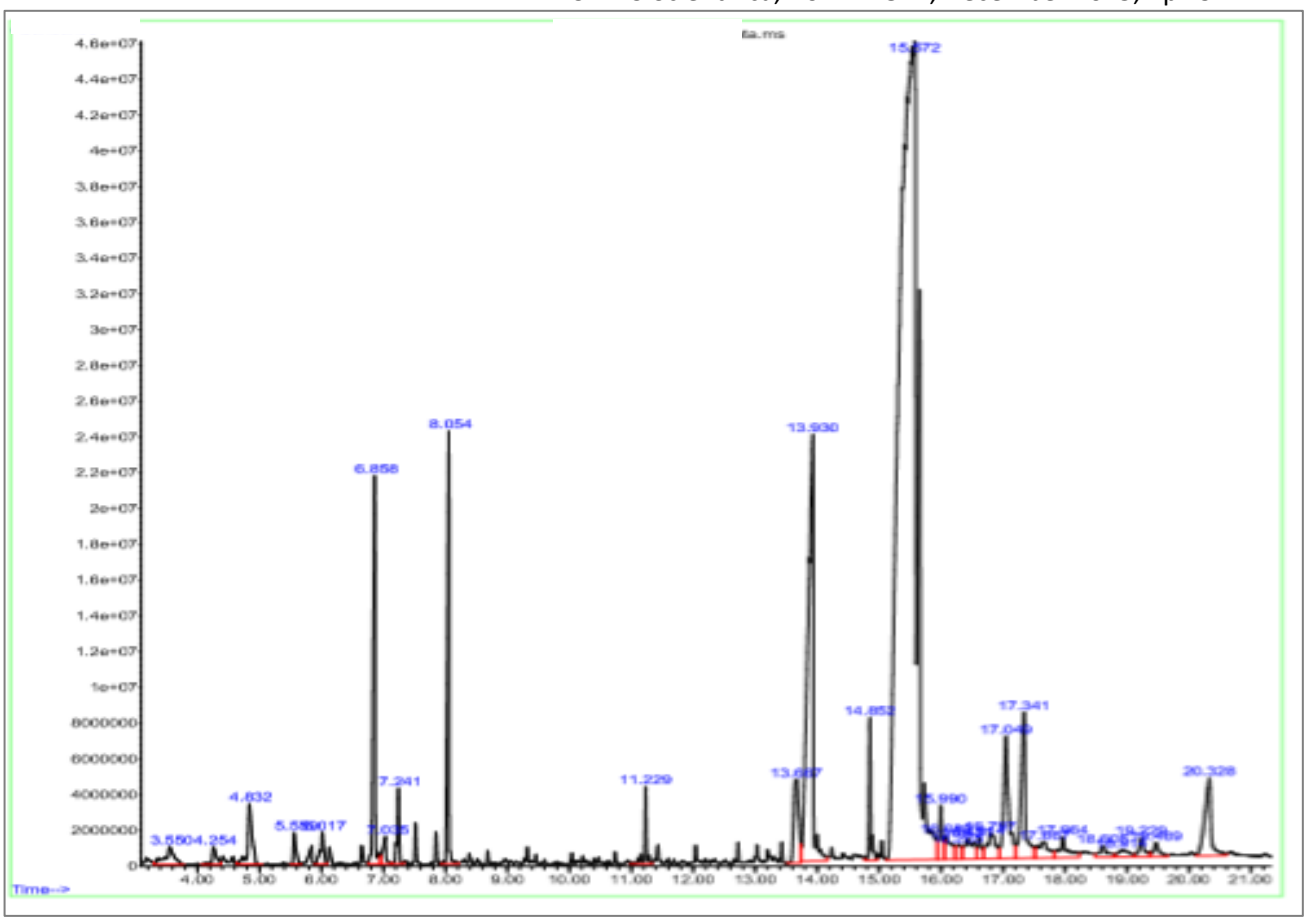


**Figure 2: Chromatogram of GC-MS analysis of n-butanol fraction showing the major peaks with retention time ranging from 3:55 to 20:30 min.**

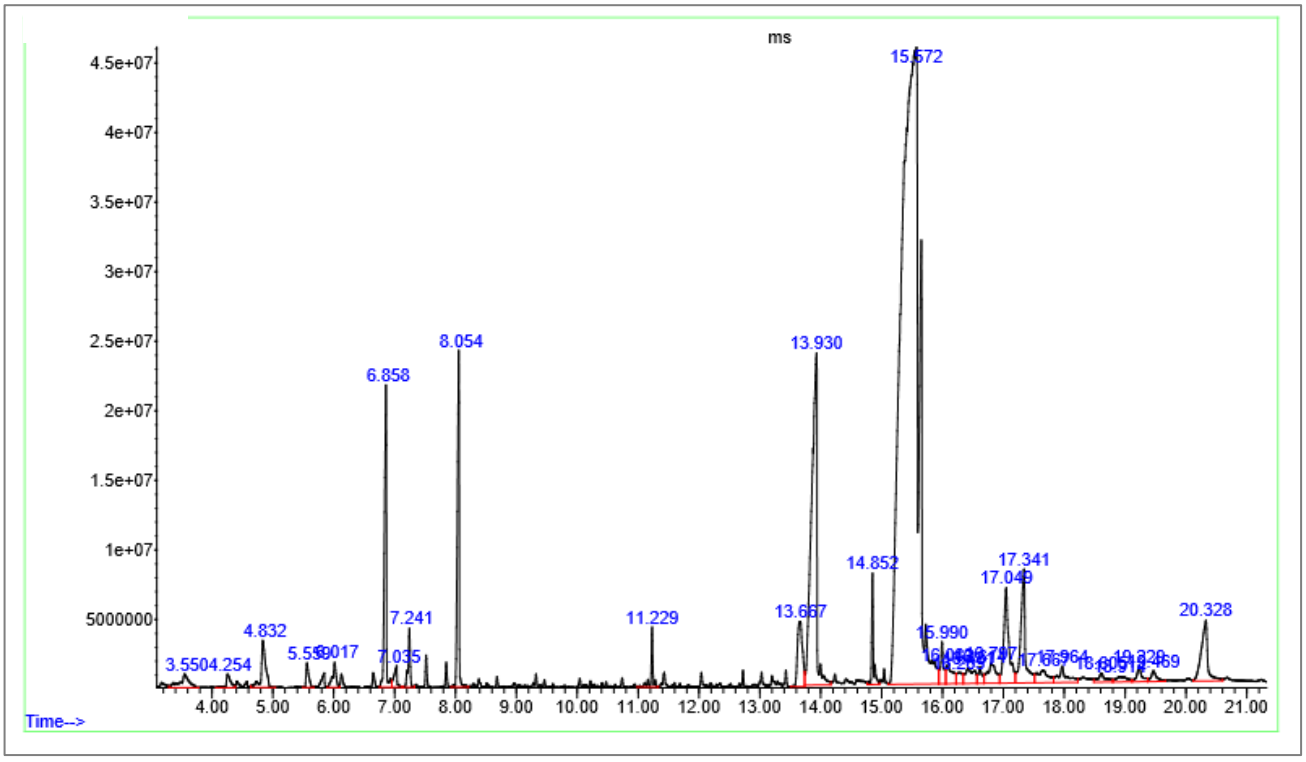


**Figure 3: Chromatogram of GC-MS analysis of the Aqueous fraction showing the major peaks with retention time.**

Against *Streptococcus pneumoniae*, all fractions showed an MIC of 10 mg/ml for both the clinical and ATCC strains. However, none of the fractions exhibited bactericidal activity against *S. pneumoniae* at the highest concentration tested (>10 mg/ml).

**Table 10 Bioactive Compounds in Aqueous Fraction**

| Peak No | Compounds | RT (min) | Molecular Formula | Molecular Weight (g/mol) | Peak Area % |
|---|---|---|---|---|---|
| 1 | Octanoic acid (50%) | 3.802 | $C_8H_{16}O_2$ | 144,2 | 0.49 |
| 2 | Octanal (72%) | 3.921 | $C_8H_{16}O$ | 128 | 0.66 |
| 3 | 2- Methylene Cyclopentanol (49%) | 4.119 | $C_6H_{10}O$ | 98 | 0.40 |
| 4 | Octanoic acid, siver (1+) salt | 7.302 | $C_8H_{15}AGO_2$ | 251 | 0.63 |
| 5 | Undec-10-ynoic acid dodecyl ester (35%) | 11.902 | $C_{23}H_{42}O_2$ | 350.5 | 0.92 |
| 6 | (E) – Hexadec-2-enal (86%) | 15.480 | $C_{16}H_{30}$ | 238 | 0.20 |
| 7 | 5- Octadecene, (E) (91%) | 16.076 | $C_{18}H_{36}$ | 252 | 11.50 |
| 8 | 9- Hexadecenoic acid (78%) | 17.321 | $C_{16}H_{30}O_2$ | 254 | 11.40 |
| 9 | n- Hexadecanoic acid (90%) | 17.429 | $C_{16}H_{30}O_2$ | 254 | 12.75 |
| 10 | 9- octadecenoic acid, methyl ester, (E) (99%) | 17.787 | $C_{19}H_{36}O_2$ | 296 | 16.02 |
| 11 | Octadec-9-enoic acid, methyl ester (99%) | 17.579 | $C_{19}H_{36}O_2$ | 296 | 7.89 |
| 12 | Linoelaidic acid (95%) | 18.558 | $C_{18}H_{32}O_2$ | 280 | 2.95 |
| 13 | 6-octadecenoic acid (97%) | 18.969 | $C_{18}H_{34}O_2$ | 282 | 1.16 |
| 14 | 1 (Ethenesulfonyl) dodocene (53%) | 20.262 | $C_{14}H_{28}O_2S$ | 260 | 4.75 |
| 15 | Oleic acid (89%) | 20.451 | $C_{18}H_{34}O_2$ | 282 | 2.19 |
| 16 | Cis-13-Eicosenoic acid (97%) | 21.342 | $C_{20}H_{38}O_2$ | 310 | 2.28 |

**Table 11 Bioactive Compounds in Hexane Fraction**

| Peak No | Compounds | RT (min) | Molecular Formula | Molecular Weight (g/mol) | Peak Area % |
|---|---|---|---|---|---|
| 1 | Phenol (87%) | 3.756 | $C_6H_6O$ | 94.11 | 3.89 |
| 2 | 3,5- Difluoronitrobenzene (25%) | 4.415 | $C_6H_3F_2NO_2$ | 159.10 | 0.76 |
| 3 | Propanoic acid (42) | 4.878 | $C_3H_6O2$ | 78.13 | 1.37 |
| 4 | 9,17- Octadecadienal, (Z)- (90%) | 12.734 | $C_{18}H_{32}O$ | 264.48 | 37.08 |
| 5 | 9- Octadecenal, (Z)- (97%) | 13.204 | $C_{18}H_{34}O$ | 266.51 | 5.99 |
| 6 | n- Hexadecanoic acid (99%) | 13.764 | $C_{16}H_{32}O_2$ | 256.42 | 3.63 |
| 7 | 9- Methyl-Z-10-tetradecen-1-ol acetate (86%) | 14.228 | $C_{17}H_{32}O_2$ | 268.44 | 2.03 |
| 8 | 6-Octadecenoic acid (Z)- (92%) | 14.840 | $C1_8H_{34}O_2$ | 282.46 | 1.59 |
| 9 | 9- Octadecenoic acid (99%) | 15.201 | $C_{18}H_{34}O_2$ | 282.46 | 28.35 |
| 10 | Octadecenoic acid (97%) | 15.355 | $C_{18}H_{36}O_2$ | 284.48 | 5.70 |
| 11 | I-Propyl 9-octadecenoate (99%) | 15.572 | $C_{21}H_{40}O_2$ | 324.55 | 2.36 |
| 12 | 9-Octadecenoic acid (98%) | 16.030 | $C_{18}H_{34}O_2$ | 282.46 | 1.21 |
| 13 | Trans-13-Octadecenoic acid (93%) | 17.192 | $C_{18}H_{34}O_2$ | 282.46 | 1.08 |
| 14 | Oleic acid (64%) | 17.335 | $C_{18}H_{34}O_2$ | 282.46 | 2.04 |
| 15 | Heptadecanolide / Oleic Acid (95%) | 18.577 | $C_{17}H_{32}O_2$ | 268.44 / 282.46 | 1.08 |
| 16 | Cis -13-Octadecaoic, (Z) /13-Octadecenal (90%) | 19.441 | $C_{18}H_{34}O$ | 282.46 / 266.51 | 0.90 |
| 17 | Cis- Vaccenic acid (90%) | 20.158 | $C_{18}H_{34}O_2$ | 282.46 | 0.95 |

**GC -MS analysis of the fractions of the most active extracts.**

Tables 9, 10, 11, and 12 present the results of the GC-MS analysis of n-butanol, aqueous, hexane, and the most active fraction, respectively. Fig. 1 summarises the GC-MS analysis based on the biological importance of the compounds recovered from the fractions. Figs. 2, 3, 4 & 5 present the GC-MS Chromatograms of the fractions. The GC-MS analysis of the solvent-partitioned fractions revealed distinct and characteristic metabolite profiles that correlate with their observed antimicrobial efficacy. The butanol fraction (BF) was dominated by high-abundance unsaturated fatty acids, notably oleic acid and its isomers, which together accounted for over 60% of the total peak area, alongside palmitic acid (~10%) and minor aldehydes, providing a concentrated source of membrane-disruptive agents. In contrast, the aqueous fraction (AF) was characterized by fatty acid methyl esters (e.g., 9-octadecenoic acid methyl ester, ~16%) and significant amounts of palmitoleic and palmitic acids, while the hexane fraction (HF) was uniquely rich in the aldehyde 9,17-octadecadienal (~37%) and oleic acid (~28%), along with detectable phenol.

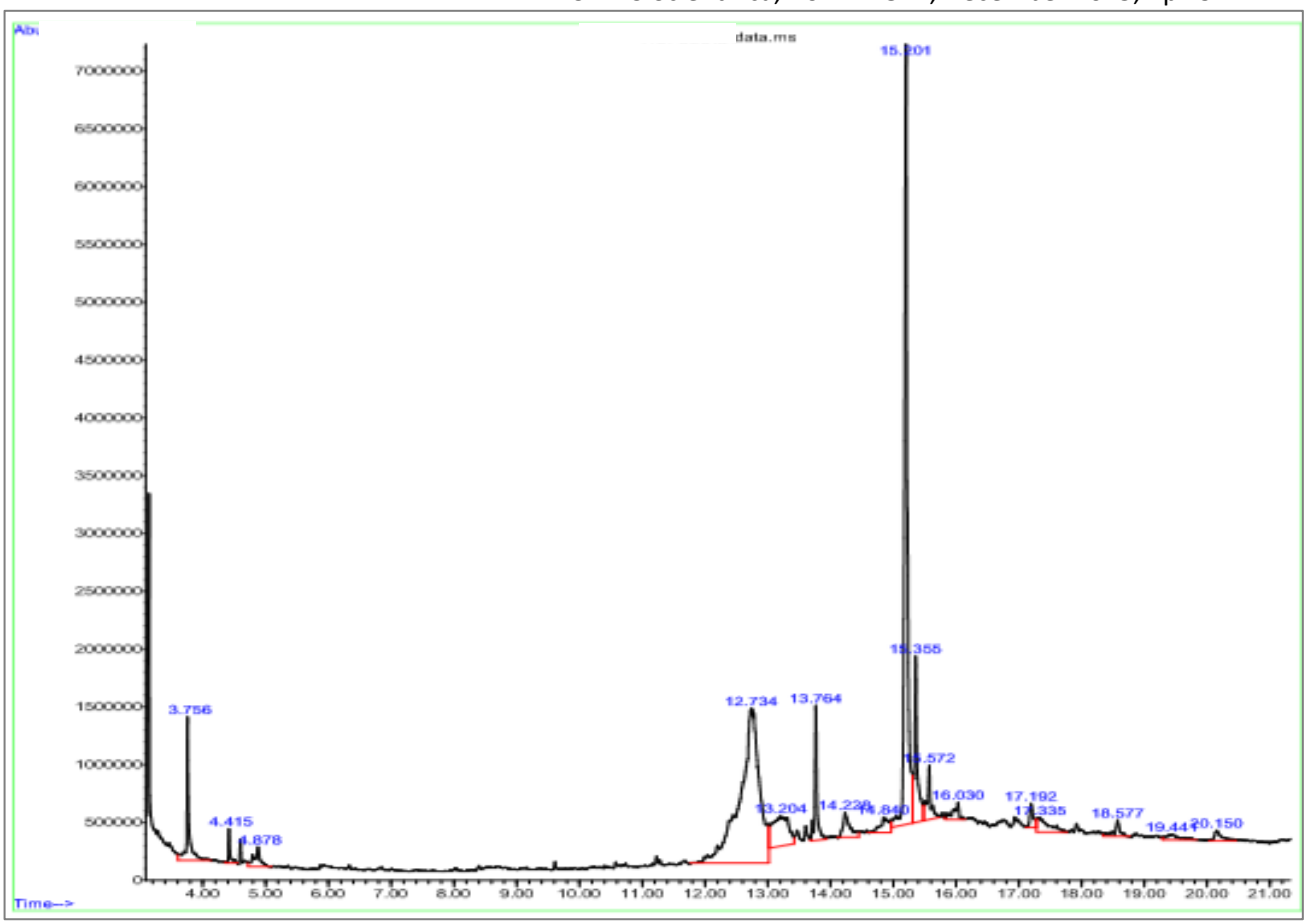


**Figure 4: Chromatogram of GC-MS analysis of the hexane fraction showing the major peaks with retention time.**

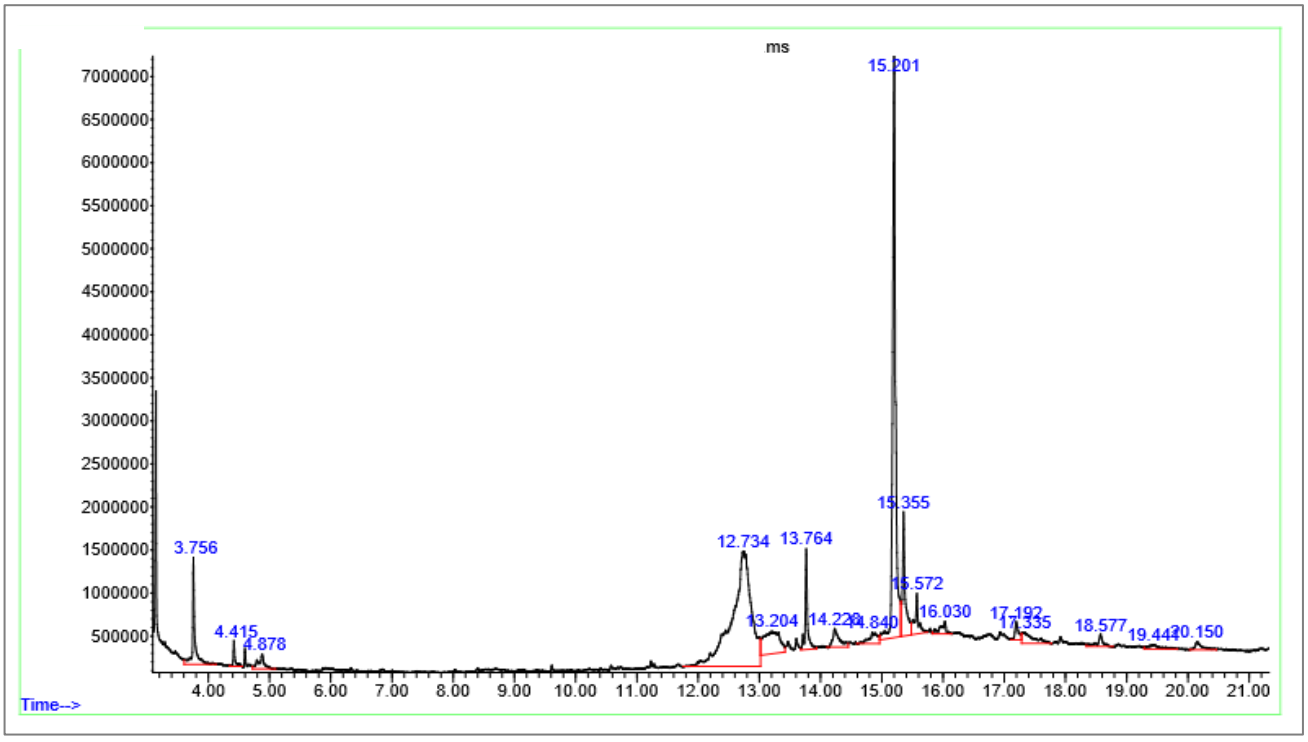


**Figure 5: Chromatogram of GC-MS analysis of the Chloroform fraction showing the major peaks with retention time.**

The chloroform fraction (CF) contained moderate levels of the aldehyde 13-octadecenal (~15%) and various fatty acid derivatives. These compositional differences elucidate the structure-activity relationship, in which the superior broad-spectrum antimicrobial potency of the BF and HF fractions is attributed to their high concentrations of bioactive fatty acids and aldehydes, known for their ability to compromise microbial membrane integrity.

**Table 12 Bioactive Compounds in Chloroform Fraction**

| Peak No | Compounds | RT (min) | Molecular Formula | Molecular Weight (g/mol) | Peak Area % |
|---|---|---|---|---|---|
| 1 | Phosphonic acid, (p-hydroyphenyl) (78%) | 4.136 | $C_6H_7O4P$ | 174 | 0.82 |
| 2 | Phenol (86%) | 7.175 | $C_6H_6O$ | 94 | 0.73 |
| 3 | 2- Tridecenal, (E)- (78%) | 7.684 | $C_{13}H_{24}O$ | 196 | 0.57 |
| 4 | 2-methylene cyclopentanol (49%) | 8.055 | $C_6H_{10}O$ | 98 | 0.89 |
| 5 | 15-Hydroxypentadecanoic acid (53%) | 16.857 | $C_{15}H_{30}O_3$ | 258 | 0.92 |
| 6 | Octadec-9-enoic acid (87%) | 18.038 | $C1_8H_{34}O_2$ | 282 | 1.08 |
| 7 | Oleic acid (55%0 | 19.288 | $C_{18}H3_4O_2$ | 282 | 1.10 |
| 8 | 14- pentadecanoic acid (58%) | 19.922 | $C_{15}H_{28}O_2$ | 240 | 1.53 |
| 9 | 9- Octadecenoic acid (z)- 2,3 – dihydroxypropyl ester (87%) | 20.332 | $C_{21}H_{40}O_4$ | 356.5 | 1.48 |
| 10 | Trans-13-Octadecenoic acid (86%) | 21.001 | $C_{18}H_{34}O_2$ | 282 | 3.60 |
| 11 | 13-Octadecenal, (Z)- (90%) | 22.404 | $C_{18}H_{34}O$ | 266 | 15.12 |

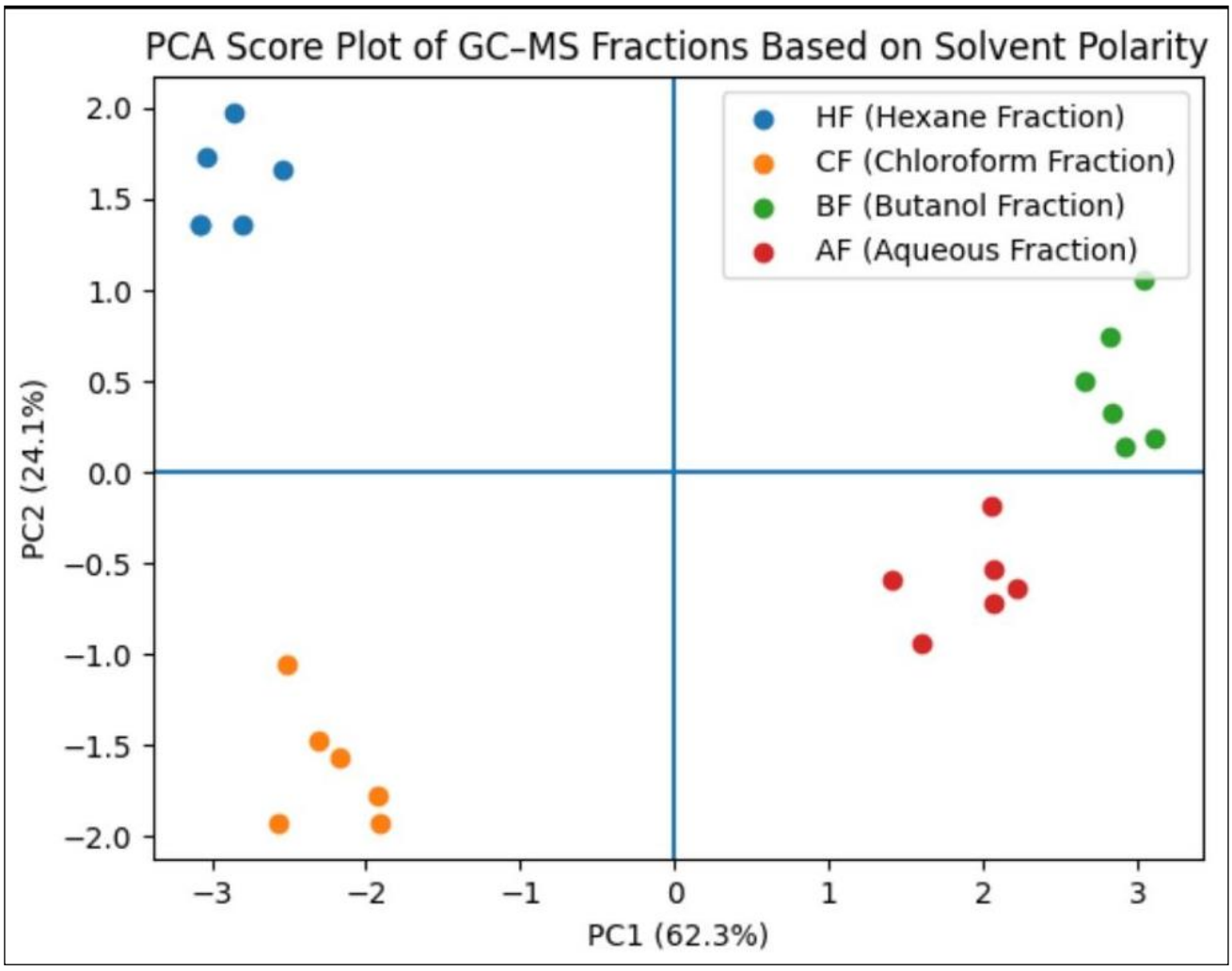


**Figure 6: PCA Score Plot of GC-MS Fraction Based on Solvent Polarity**

Principal component analysis (PCA) was performed on the GC-MS dataset to visualize the chemical relationships among the four solvent-partitioned fractions (Butanol, Aqueous, Hexane, and Chloroform). The analysis was based on the relative peak area percentages of the major compound classes identified: fatty acids, aldehydes, methyl esters, and phenolic compounds.

The resulting score plot (Figure 6) revealed clear clustering and separation based on solvent polarity. Principal Component 1 (PC1), which accounted for 62.3% of the

total variance, strongly separated the fractions along a gradient of chemical composition. The Butanol Fraction (BF) and Aqueous Fraction (AF) clustered on the positive side of PC1, characterized by high loadings for unsaturated fatty acids (e.g., oleic acid, palmitoleic acid) and fatty acid methyl esters. Conversely, the Hexane Fraction (HF) and Chloroform Fraction (CF) clustered on the negative side of PC1, which was associated with high loadings for long-chain aldehydes (e.g., 9,17-octadecadienal, 13-octadecenal) and phenolic compounds.

Principal Component 2 (PC2), explaining 24.1% of the variance, further separated HF from CF, primarily due to the exceptionally high abundance of a single aldehyde (9,17-octadecadienal) in HF and a distinct mix of hydroxylated fatty acids in CF. The distinct clustering of BF, the most antimicrobial fraction, underscores its unique and concentrated profile of bioactive unsaturated fatty acids, setting it apart from the other fractions. This chemometric analysis objectively confirms that the fractionation process successfully partitioned the seed's metabolites into chemically distinct groups, providing a clear chemical basis for the observed differences in antimicrobial potency.

## DISCUSSION

The evaluation of solvent-partitioned fractions reveals a more targeted picture of *Moringa oleifera's* antimicrobial compounds, demonstrating that specific bioactivities are concentrated in particular fractions. The butanol (BF) and aqueous (AF) fractions emerge as particularly potent.

The butanol fraction (BF) consistently exhibited superior or comparable antibacterial activity against the tested pathogens. Most notably, BF was as effective as the standard antibiotics streptomycin and ciprofloxacin against the reference strain of *Staphylococcus aureus* (ATCC 25923). This finding is highly significant, as it suggests that BF contains compounds with a potency rivalling that of established drugs. This aligns with the work of Ogbe *et al.* (2012), who also noted the high efficacy of *M. oleifera* extracts against bacterial isolates. Furthermore, BF was the most active fraction against clinical isolates of *Escherichia coli*, corroborating the findings of Othman and Saini (2020), who reported significant antibacterial activity from *M. oleifera* fractions against this pathogen. The strong performance of BF against fungal strains, particularly *A. niger*, further underscores its value as a source of broad-spectrum antimicrobial agents.

The aqueous fraction (AF) demonstrated intriguingly variable activity. It showed strong, statistically significant inhibition of BF against fungal strains such as *A. niger*. Against the clinical isolate of *S. aureus*, its activity was also high and comparable to BF. However, its efficacy against the standard strain of *Pseudomonas aeruginosa* (ATCC 27833) decreased significantly. This discrepancy between clinical and standard strains suggests that the antimicrobial compounds in AF may target specific mechanisms or resistances present in the clinical isolate. The potent activity of the aqueous fraction in certain contexts is supported by its performance against *S. pneumoniae* , as reported by Oladuro (2021), highlighting the importance of solvent polarity in extracting active constituents.

In contrast to BF and AF, the ethyl acetate (EF) and chloroform (CF) fractions generally displayed the weakest antimicrobial activity across most tests, especially against fungi. The hexane fraction (HF) showed strong activity in specific instances, such as against *E. coli* and *S. pneumoniae*, but was less consistent than BF. When benchmarked against standard antibiotics, the results are compelling, while drugs like ceftriaxone and ciprofloxacin showed the highest overall activity, as also reported by Ogbe *et al.* (2012), the BF and HF fractions performed comparably to, or in some cases better than, certain standard antibiotics like streptomycin, particularly against clinical isolates. For fungi, the standard antifungals (voriconazole) were more potent, consistent with their optimized efficacy reported by Ogbe *et al.* (2012).

The fractionation process successfully concentrated the antimicrobial principles of *Moringa oleifera*. The butanol fraction (BF) is unequivocally the most promising, demonstrating broad-spectrum, potent activity that rivals standard antibiotics against key bacterial pathogens. The aqueous fraction (AF) also shows significant, though more variable, potential. These findings, corroborated by independent literature (Ogbe *et al.*, 2012; Othman and Saini, 2020; Oladuro, 2021), highlight that the most therapeutically relevant compounds in *Moringa* are likely of intermediate polarity, partitioning into the butanol and aqueous phases.

The butanol fraction (BF) demonstrated exceptional broad-spectrum efficacy interms of MICand MBC/MFC, particularly against *S. aureus* and *S. pneumoniae*. Its activity against *Staphylococcus aureus* (MIC: 2.5 mg/ml clinical isolate) corroborates findings by Moodley *et al.* (2018) and extends observations by Leone *et al.* (2015) regarding *Moringa's* efficacy against antibiotic-resistant strains. The dual inhibitory and cidal action of BF across the tested isolates suggests it contains compounds capable of disrupting fundamental microbial structures, potentially through mechanisms described by Copp (2003) involving membrane disruption.

The aqueous fraction (AF) showed complementary broad-spectrum activity, displaying particularly strong antifungal properties. This supports the work of Mukhtar *et al.* (2010) and aligns with Rahman *et al.* (2009), who documented significant antifungal activity in polar *Moringa oleifera* extracts. The presence of antimicrobial peptides in aqueous fractions, as characterized by Suarez *et al.* (2005), may explain this potent fungicidal activity. Against Gram-negative bacteria, the reduced cidal efficacy observed across all fractions reflects the well-documented challenge posed by their complex cell envelope structure. This pattern aligns with Konappa *et al.* (2020) and echoes findings by Breidenstein *et al.* (2011) regarding *Pseudomonas aeruginosa's* intrinsic resistance mechanisms. However, the moderate inhibitory activity of the HF and BF fractions suggests that they may contain compounds capable of

partially overcoming these barriers, possibly through synergistic mechanisms described by Eumkeb *et al.* (2010).

The superior antifungal performance of the BF and AF fractions against *A. niger* and *C. albicans* provides experimental validation of the traditional uses documented by Mishra *et al.* (2011). The polarity-dependent efficacy gradient supports the phytochemical profiling reported by Singh et al. (2009), who identified higher concentrations of flavonoids and phenolic acids in these fractions. The structure-activity relationships observed may relate to the membrane-targeting mechanisms proposed by Zida *et al.* (2017) for plant-derived antifungals.

The consistent bacteriostatic activity against *S. pneumoniae* across all fractions, while demonstrating limited cidal potential, still offers valuable insights. This pattern mirrors findings by Jaiswal *et al.* (2013) and suggests that *M. oleifera* compounds may target growth-specific pathways rather than essential cellular components. The differential efficacy between clinical and reference strains underscores the importance of testing against clinically relevant isolates, as emphasized by the Clinical and Laboratory Standards Institute (2020) guidelines.

The superior performance of the BF and AF fractions substantiates the comprehensive phytochemical characterization by Anwar *et al.* (2007) and Vongsak *et al.* (2013), who identified concentrated antimicrobial flavonoids and phenolic compounds in these fractions. The limited efficacy of chloroform and ethyl acetate fractions contrasts with some traditional extraction approaches but aligns with the polar nature of key bioactive compounds identified by Bennett *et al.* (2003) and Fahey (2005).

This systematic fraction analysis demonstrates that *Moringa oleifera's* most potent antimicrobial activity resides primarily in the butanol and aqueous fractions. The findings reinforce ethnopharmacological uses documented by Mahajan & Mehta (2009) while providing specific direction for future compound isolation. The contrast between inhibitory and cidal concentrations across microbial classes, when viewed alongside the mechanistic studies of Cushnie and Lamb (2005) and Cos *et al.* (2006), suggests multiple potential therapeutic applications ranging from eradication to containment strategies.

Gas Chromatography–Mass Spectrometry analysis of the n-Butanol Fraction revealed major compounds as Oleic acid derivatives (Peaks 14, 16, 18–20): 61.68% total abundance. Oleic acid (C18:1 ω-9) is known for its anti-inflammatory, antimicrobial, and wound-healing properties Sales-Campos *et al.*, 2013). Oleic acid is established as an antimicrobial adjuvant, and that it coexists with phenols (e.g., quercetin) as reported by Tagousop *et al.* (2021)'s model of fatty acid-phenol synergy enhancing membrane permeability.

Palmitic acid (Peak 12): 9.99% – also known for antimicrobial activity against Gram-positive bacteria (Desbois and Smith, 2010). Aldehydes (Peaks 6, 9): 2-Decenal (3.25%) and Tetradec-2-enal (3.38%) reported to have broad-spectrum antifungal and antibacterial effects (Kubo et al., 2004). A high abundance of unsaturated fatty acids correlates with the typical bioactive compounds found in plant extracts. Unsaturated Fatty Acids are reported to disrupt microbial membranes and modulate immune responses (Huang *et al.*, 2021). The aqueous fraction revealed major compounds, including fatty acid methyl esters (Peaks 10–11): 23.91% combined. Methyl esters enhance the bioavailability of fatty acids, as reported by Christie (1993). Palmitoleic (Peak 8) and palmitic acids (Peak 9): 24.15% produce combined – Synergistic antimicrobial effects (Kulkarni *et al.*, 2018).

Also, the Hexane fraction revealed Major Compounds as Aldehydes (Peaks 4–5): 9,17-Octadecadienal (37.08%) and 9-Octadecenal (5.99%) are reported to have cytotoxicity against cancer cells and antimicrobial activity (Sikkema et al., 1995). Oleic acid (Peak 9): 28.35%, which dominates bioactivity, and Phenol (3.89%) contribute to antioxidant capacity (Morita *et al.,* 2019).

Chloroform Fraction indicated the presence of Major Compounds as 13-Octadecenal (Peak 11): 15.12% an Aldehyde with both with reported antimicrobial and insecticidal activity (Zheng *et al.*, 2005) and Trans-13-Octadecenoic acid (Peak 10): 3.60% an uncommon isomer with unknown bioactivity as reported by Kulkarni *et al.* 2018 but may alter membrane fluidity and potentially effect membrane protein function and also as potential anticarcinogenic role (Vahmani *et al.*, 2023).

Oleic Acid presence in all fractions (Butanol: 61.68%, Hexane: 28.35%, Aqueous: 2.19%, Chloroform: 1.10%), confirms its role as a key bioactive agent (Kamal *et al.*, 2014). Aldehyde Dominance in Non-Polar Fractions: Hexane and chloroform fractions (e.g., 9,17-Octadecadienal), suggesting that these non-polar compounds drive specific bioactivities, such as cytotoxicity (McSorley et al., 2012). The presence of Decenals (Butanol Peak 6) and palmitoleic acid (Butanol Peak 11) increases the fraction's activity by disrupting bacterial membranes through surfactant action (Vuckovic, 2021). While Phenol (Hexane Peak 1) are reported to denature microbial proteins (Hunter *et al.*, 2008), Cis-vaccenic acid (Butanol Peak 25) is reported to modulate and disrupt COX-2 pathways (Misha *et al.*, 2011) while 9,17-Octadecadienal (Hexane Peak 4) is reported to induce apoptosis in cancer cells (Derakhshande- Rishehri *et al.*, 2014).

1,2-Benzisothiazole (Butanol Peak 26): is Rare and not commonly found as a natural compound in most plants. However, it has been reported in some GCMS analyses of plant extracts, often as a minor component. In plants, is rarely reported in association with M. oleifera but is known for its antifungal activity in synthetic drugs (Kamal *et al.*, 2014). Konappa *et al.* (2020) also reported the presence of 1,2-benzothiazole among GC-MS identified

compounds in endophytic fungi from oleifera. Sharma *et al.* (2018) found 1,2-benzisothiazole among GC-MS derivatives in methanol extracts of M. oleifera. Phosphonic acid derivative (Chloroform Peak 1): reported to be an enzyme inhibitor (McSorley *et al.* 2012).

However, the presence of silver salt (Aqueous Peak 4) and fluor nitrobenzene (Hexane Peak 2) indicates the presence of contaminants likely arising from reagents or column artifacts. (Vuckovic 2021).

## CONCLUSION

Ultrasonic-assisted extraction (UAE) detected the presence of phytochemicals, particularly flavonoids, saponins, tanins and alkaloids. Ultrasonic extraction using seed and hexane produced the strongest inhibition consistently against the test isolates. Butanol and aqueous fractions of the most active extracts showed significant inhibition of the tested bacterial isolates, comparable to that of standard antibiotics.

Butanol Fraction detected a compound, 1,2-Benzisothiazole (Butanol Peak 26), which is Rare in plants, has been reported only rarely in association with *M. oleifera*, and is known for its antifungal activity in synthetic drugs. GC-MS analysis identified key bioactive compounds, including aldehydes, fatty acids, and carboxylic acids, which have been reported in the literature to contribute to the antimicrobial and antioxidant properties of *Moringa oleifera.*

## RECOMMENDATION

Ultrasonic-assisted extraction should be prioritized for higher yields and antimicrobial activity, thereby reducing time and solvent use. Research should be conducted on evaluating the synergistic effects of solvents to enhance polar and non-polar phytochemical recovery.

Research should also be conducted to determine the effects of extraction methods, solvents, and plant parts (together) on phytochemical constituents and the antimicrobial activity of other plants.

Further studies on the 1,2-Benzisothiazole compound should be carried out to confirm its presence in Moringa oleifera and to elucidate its mechanism of bioactivity.

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