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In Vitro Antimicrobial Activity of the Oil Obtained from the Seeds of Citrullus Colocynths (Bitter Apple)

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ABSTRACT

The phytochemical and antimicrobial analysis of the crude extract of oil gotten from the seeds of Citrullus colocynths were carried out using standard methods. The result of the photochemical screening showed that the oil extract of Citrullus colocynths contains alkaloids, flavonoids, saponins, tannins, terpenoids and anthraquinone. The bacterial activity of Citrullus colocynths oil extract was assessed against a panel of pathogenic bacteria such as Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) results show that the microorganisms show zone of inhibition of 4.5 mm for Escherichia coli, 4.6 mm for Pseudomonas aeruginosa, 5.9 mm for Salmonella typhi and 6.1 mm for Staphylococcus aureus at a concentration of 200 mg/ml, 400 mg/ml and 600 mg/ml. Notably, Staphylococcus aureus and Pseudomonas aeruginosa exhibited larger zones of inhibition compared to Escherichia coli and Salmonella typhi. Thin Layer Chromatography (TLC) revealed three spots of the isolated compound, while MIC and MBC/MFC values were also determined with a concentration of 600 to 700 µg/cm^3. Synergistic effects were assessed in combination with standard antibiotics, and mechanisms of action were investigated through chemical analysis. The research uncovered the antimicrobial potential of Citrullus colocynths seed oil, offering promising insights for further exploration and potential clinical applications. The FTIR spectrum of the isolated compound revealed characteristic peaks, including a strong absorption at 2855.1 cm-1 indicative of alkanes (C-H) and another at 3011.7 cm1 for C=C in aromatics. The presence of a broad absorption at 2922.2 cm-1 suggested the presence of C-H groups, consistent with a reported absorption at 3420 cm-1. Compound gotten from the GCMS analysis which include 1-Decene and Mesitylene have been used in the synthesis of medicine such as asthma, cancer, tuberculosis and jaundices for their antimicrobial and anti-inflammatory properties.

Keywords: Phytochemicals, minimum inhibitory Concentration, antimicrobial, C*itrullus colocynthis,* Thin Layer Chromatography.

INTRODUCTION

Citrullus colocynthis is generally referred to as the bitter cucumber, desert gourd, or colocynth. It is a plant species which belong to the family of Cucurbitaceae. It is commonly known as Tangir in Yoruba, Batsa or Batsamiya in Hausa and Agboh mmuo or Akpunsi in Igbo. It is native to various regions of Africa, the Middle East, and southern Europe. The plant has a distinctive feature, with round, yellowish-green fruits that resemble small watermelons [7]. The main aim of this research is to determine in vitro antimicrobial efficacy of the oil from the seeds of Citrullus colocynthis commonly known as bitter apple.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

Citrullus colocynthis is a member of the Cucurbitaceae family. It is extremely bitter and generally known for its medicinal and nutritional advantage as well as its ability to cure cancer and diabetic. The seed oil of the plant also serves as analgesic, purgative and laxatives. It has different kinds of compounds present in it. Some of it are; resins, flavonoids, alkaloids, steroids, gums, glycosides, Citrullus and cucurbitacin glucoside [15]. It has been used widely in several countries such as in the hot and subtropical areas to treat diabetes and also hypertension in the United Arab Emirate (UAE). It is used in the Mediterranean countries as an anti-inflammatory drug and as a purgative antirheumatic drug in Saudi Arabia. In some other countries, it can be used to treat urinary tract disorder. The fruits and seeds of Citrullus colocynthis has been used for the treatment of diabetes, ulcer, jaundice, asthma, sore throat, elephantiasis and tuberculosis, while the roots are majorly used to treat arthritis, cough, boils, joints inflammation and skin diseases [16]

Figure 1; Image of Citrullus colocynthis

Source: https://upload.wikimedia.org/wikipedia/commons/3/3b/Citrullus_colocynthis_004.JPG

On lengthy petioles, the angular leaves are arranged sporadically. Every leaf contains three to seven lobes and measures approximately five to ten centimeters in length. The other lobe of the plant may occasionally have an ovate shape. The leaves are trapezoidal in form and have several clefts. The leaves have open sinuses and a rough, hairy feel. The leaves have a beautiful green color on their upper surface and a rather pale lower surface. About 15 to 30 globular fruits with a diameter of approximately 7 to 10 centimeters are produced by each bitter apple plant. The fruit's exterior is coated with a green skin with yellow stripes. The fruits could also have a yellow hue. The ripe fruits have a firm, thin skin that rind. The fruits contain several ovate compressed seeds inside a delicate, white flesh. The yellow flowers are solitary and occur in the leaf axils. Since the pistils and stamens are present in several blooms on the same plant, they are monoecious. Their peduncles are lengthy. A yellow campanulate is also a component of every bloom. The calyx is divided into five parts, while the corolla contains five lobes. The hairy, villous ovary of the female flowers makes them clearly distinguishable from the males. The seeds are smooth, compressed, ovoid-shaped, and measure about 6 mm in size. On the parietal placenta is where they are found. The hue of the seed’s ranges from pale yellowish-orange to dark brown. The enormous perennial root of the bitter apple plant produces long, thin, angular, harsh, scratchy stalks that resemble vines. Usually lying flat on the ground, the stems tend to clamber over shrubs and herbs with their tendrils that branch axillary. Both wild and farmed Citrullus colocynthis can be found across Ceylon and India. Additionally, it is native to the Mediterranean region, tropical Africa, west Asia, and Arabia. The fruits cure tumors, ascites, leucoderma, ulcers, asthma, bronchitis, urinary discharges, jaundice, enlargement of the spleen, tuberculosis glands of the neck, dyspepsia, constipation, anemia, throat diseases, elephantiasis, and joint pain. They are also bitter, pungent, cooling, purgative, anthelmintic, antipyretic, and carminative. Root is helpful in treating rheumatism, jaundice, ascites, urinary tract infections, belly enlargements, children's coughs, and asthma attacks. a root poultice that helps with breast inflammation. Fruit or root, either combined or separately are used in curing boils and pimples and a concoction of it is rubbed into a paste with water. The part of the root is applied to children's expanding abdomens [3]. Phytochemical compounds, also known as phytochemicals or secondary metabolites, are naturally occurring chemical compounds produced by plants as a part of their metabolic

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processes. These compounds play essential roles in plant growth, development, and defines against environmental stresses. Moreover, many of these compounds have demonstrated significant health benefits for humans, leading to increased interest in their study and potential applications in medicine and other industries. Phytochemical compounds include: Flavonoids: A diverse group of polyphenolic compounds, flavonoids are known for their antioxidant properties. They are found in fruits, vegetables, tea, and various medicinal plants. Examples include quercetin, kaempferol, and catechins. Alkaloids: Nitrogen-containing compounds with diverse pharmacological activities. Examples include morphine (from poppies), caffeine (from coffee beans), and quinine (from cinchona bark). Terpenoids: Derived from isoprene units, terpenoids include essential oils and compounds with varied biological activities. Examples include menthol, found in mint, and artemisinin, an antimalarial compound. Glucosinolates: Common in cruciferous vegetables, glucosinolates are sulphur-containing compounds with potential anticancer properties. Examples include sulforaphane in broccoli. Carotenoids: Responsible for the red, orange, and yellow pigments in fruits and vegetables, carotenoids have antioxidant properties. Examples include beta-carotene, lycopene and lutein [14].

Antibiotic-resistant bacteria have made the hunt for potent antimicrobial drugs more important than ever. Plant extracts have long been used in traditional medicine due to their antibacterial qualities and new research has provided strong support for this theory by demonstrating the extracts' effectiveness. Citrullus colocynthis, often known as the Bitter Apple, is one of such plants whose potential in a range of medicinal applications has been recognized. But in addition to the plant extracts, there had been a growing interest in the nanoparticles made from other plants extracts, which have demonstrated potential as new antibacterial agents. Plant-derived nanoparticles are effective antibacterial agents, as evidenced by recent study. For example, a study by Li et al. (2022) revealed that plant extract-derived silver nanoparticles had strong antibacterial activity [18]. Furthermore, Dhanasekaran et al.'s work from 2021 emphasizes the antibacterial and antifungal properties of nanoparticles made from different plant sources [9]. These results highlight the effectiveness of plant-derived nanoparticles as a competitive substitute for traditional antibiotics. This is further corroborated by the discovery made by Choi et al. (2020) that nanoparticles made from plant extracts have strong antibacterial qualities against a variety of diseases [7]. In a similar vein, Zhang et al.'s study from 2022 demonstrated the strong antibacterial activity of nanoparticles made from plant extracts, highlighting their potential to fight germs that are resistant to many drugs [38]. The application of plant-based nanoparticles is also consistent with the results of Ali et al. (2020), who showed how beneficial these nanoparticles are [1]. Apart from nanoparticles, the antibacterial effects of plant extracts have been thoroughly investigated. De Souza et al.'s research from 2021, for instance, gives a summary of the antibacterial effectiveness of different plant extracts, highlighting their potential as medicinal agents [8]. This idea is further supported by the review by Pal et al. (2021), which emphasizes the plant extracts' broad-spectrum antibacterial activity against various microbial strains [28]. Additionally, Lee et al.'s study from 2022 looked at how plant extracts and manufactured antibiotics worked together, and it showed promise for raising antimicrobial efficacy [17]. In light of this, the main objective of our research is to determine the oil extracted from Citrullus colocynthis seeds' in vitro antibacterial activity. The purpose of this study is to add to the expanding body of knowledge on plant-derived antimicrobial agents and investigate the possibility of using bitter apple seeds to synthesize nanoparticles or extracts that have powerful antibacterial properties.

MATERIALS AND METHODS

Sample Collection and Authentication

The fruit of Citrullus colocynthis was collected by first removing the seeds from the fruits and was taken as the sample. Sample collection was done at Ilorin Central Market, Ilorin West Local Government Area of Kwara State, Nigeria in May 2023. The plant sample was authenticated at the Herbarium Section of the Department of Biological Sciences, Nigeria Defence Academy Kaduna, with voucher number (NDA/BIOH/2023/18). The fresh samples were airdried for two weeks, pulverized into a fine powder and stored in a glass container until further use [25]

Extraction of oil from the seed

The extraction was carried out using the method of Soxhlet in the ratio 1:3 (w/v). 100g of ground Citrullus colocynthis was measured into a porous bag (thimble) made of clean cloth and sealed, after which it was placed

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in the sample chamber of the Soxhlet apparatus. Subsequently, it underwent extraction with 300ml of n-hexane continuously for a duration of 3 hours at a temperature of 60 degrees Celsius. Following this, the solvent was recovered, and the obtained oil was transferred to a beaker positioned on a water bath for a period of 1 hour. The resulting oil was then weighed to determine the yield. Subsequent to the yield calculation, assessments of oil quality parameters were conducted using 100g of the initial weighed sample and 33.5mL of the oil extract [24]. Figure 2 below is a schematic illustration showing the mode of action of the oil extract and the diagram highlights how the oil extract destroys the structures of bacteria, including the cell membrane and cell wall, which leads to cellular content leakage, enzyme inhibition and interference with Deoxyribonucleic acid (DNA) replication.

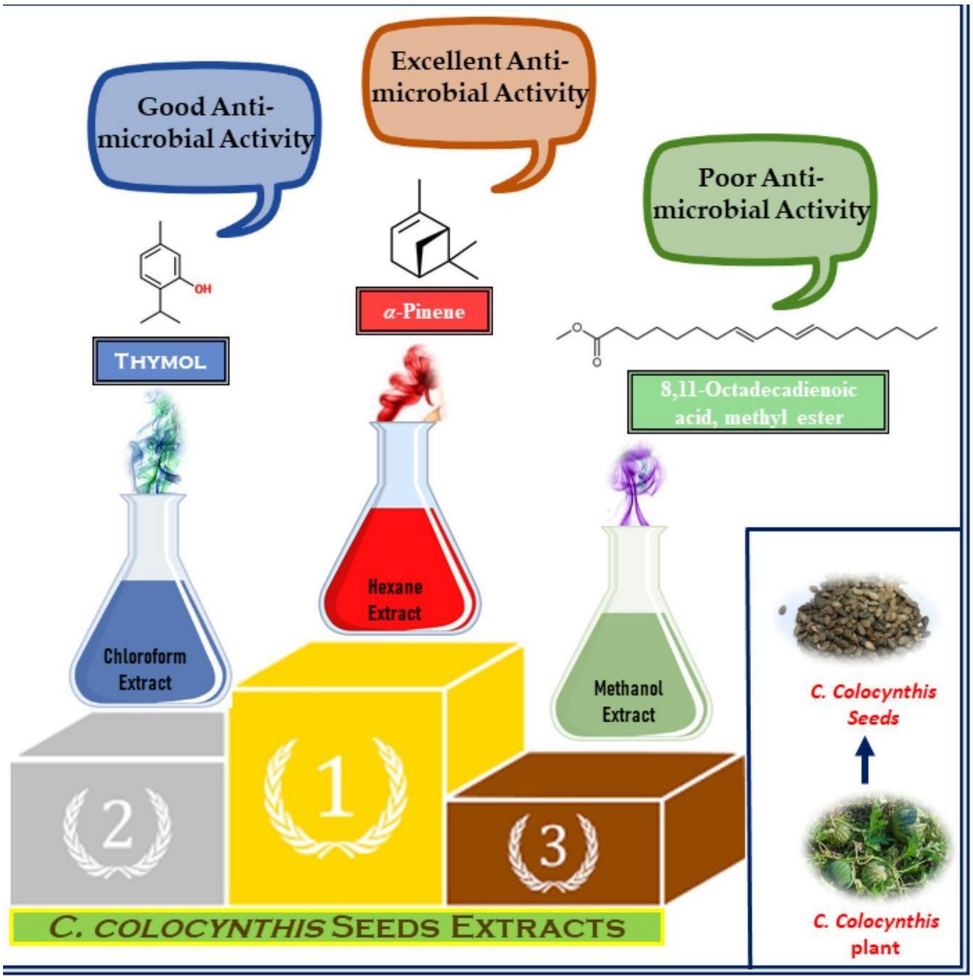


Figure 2; Schematic illustration of the mode of action of the oil extract of Citrullus colocynthis

https://pub.mdpi-res.com/plants/plants-12-00567/article_deploy/html/images/plants-12-00567-ag.png?1675311823

Extraction Yield was calculated by using equation 1

$$\frac{\text{Weight of extract (g)}}{\text{Weight of sample (g)}} \times \frac{100}{1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(i)}$$

Phytochemical Analysis;

The presence of bioactive compounds in the n-hexane oil extract of the seeds were determined using standard methods as described by [25]

Thin Layer Chromatography (TLC) Analysis

Precoated silica gel of the Thin Layer Chromatographic plates was used to determine the separation profile of

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

the oil extracts using one-way ascending technique. Capillary tube was used to manually apply spots on the TLC plate and the chromatogram was developed in an airtight chromatographic tank at room temperature employing different solvent systems. The spots were visualized using iodine crystals for 5 minutes [25].

Retention Factor (Rf) values were calculated using Equation (ii).

$$R_f = \frac{\text{Distance moved by sample}}{\text{Distance moved by solvent}} \quad \text{..... (ii)}$$

Antimicrobial Activity Test (Agar Well Diffusion Method)

Test Organisms

The organisms used in this research work were Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, and Candida albicans obtained were the from Microbiological Unit at National Agency for Food and Drug, Administration and Control (NAFDAC) Kaduna State, Nigeria. The bacterial cultures were checked, grown on Tryptone Soya Agar slant while the fungi were grown on a slant of Sabouraud Dextrose Agar (SDA) [25].

Microbial Growth Media Preparation

The manufacturer's instructions for preparing culture media were followed. 38 grams of Tryptone Soya Agar was weighed, combined with 1000 cm^3 of distilled water, and autoclaved at 120 ℃ for 20 minutes under 1 bar of pressure to sterilize it. The medium was then dispensed into petri dishes to produce a uniform depth. The culture media was allowed to cool at room temperature without being disturbed until it becomes gel (solidified). Then, it was sterilized for 24 hours in an inverted manner in an incubator at 37. Then, plastic bags were used to preserve the prepared plates between 4 and 8 degrees Celsius. Agar wells were created in the medium using 6 mm corkborer. 0.1 ml of the broth culture with the test microorganisms were introduced into the plates. Sterile cotton swab was employed to evenly distribute the inoculums over the surface of the medium and the plates were allowed to stand for 5 mins. Tryptone Soya Agar was used for Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus while Sabouraud Dextrose Agar was used for Candida albicans [25]

Determination of Antibacterial Activity

Three concentrations of Citrullus colocynthis oil extract were prepared (200mg/ml, 400mg/ml, and 600mg/ml). Furthermore, 200mg/ml of Ciprofloxacin (Positive control) was prepared and each concentration of the extracts was added to the antibiotics and also tested on the organisms. The antibiotic was introduced into separate well as controls. Each plate had three wells. 0.1 mL of the extracts, the extracts plus (+) antibiotics, and antibiotics were introduced into the properly labelled wells in the medium. The plates were also incubated at 26.4℃ for 24hrs. Antibacterial activity was determined by measuring the diameter of zone of exhibition in millimetres around the wells with a standard ruler [10].

Determination of Antifungal Activity

The standard inoculum (0.1 mL, 0.5 McFarland turbidity standard = 1.0 x 10^8 cfu/mL) of the test fungi was spread into two sterile Sabouraud Dextrose Agar (SDA) plates so as to achieve even growth. The plate was allowed to dry and a sterile cork borer (8.0 mm diameter) was used to bore holes aseptically in the agar plates. The extract was prepared and serially diluted using 10% dimethylsulphoxide (DMSO) to achieve different concentrations of 25, 50, 100 and 200 mg/mL.

Subsequently, 200 µL of each concentration of the extracts was introduced into the bored wells. At 37 ℃ for 48 hours, the extract was allowed to diffuse into the medium in an incubator. As a positive control, 5 mg/mL was prepared and poured into the single hole in the middle of one of the petri dishes. Antifungal activity of the extracts was determined by measurement of zones of inhibition produced around the wells. The test fungi's level of susceptibility was evaluated by the zones' diameter [13, 29]. 200 mg/mL of Fluconazole was used as the

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

positive control for the antifungal activity.

Broth Dilution Method for the Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the n-hexane seed oil extract.

Preparation of Serial Dilutions

Serial dilutions of the n-hexane oil extract of Citrullus colocynthis was prepared by creating a series of decreasing concentrations of the extract in a sterile broth medium. The extract was diluted to obtain concentrations of 8, 7, 6, 5, and 4 x 10^2 µg/cm³.

Preparation of the Inoculum

The test organisms (Escherichia coli, Salmonella typhi, Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans) were suspended in a standardized broth which was prepared according the standardized method described by [12]. The suspension was standardized to match a 0.5 McFarland standard, ensuring a uniform bacterial load (approximately 1-2 x 10^6 CFU/ml). The fungal suspension of Candida albicans was similarly standardized.

Inoculation of Microtiter Plates

Each dilution of the oil extract was dispensed into a separate well of a microtiter plate or test tubes. A small volume of the standardized inoculum (approximately 100 µl) of each microorganism was added to the corresponding wells or tubes containing the oil extract. This results in a final mixture of extract and microorganisms, where the oil extract interacted with the microorganisms over time.

Incubation

The microtiter plates or test tubes were covered and incubated at 35-37°C for 18-24 hours for the bacteria and at a temperature of 28-30°C for the fungal strain, *Candida albicans*. During this incubation period.

Characterization

Characterization of the isolated compound was carried out using Gas Chromatography Mass Spectroscopy (GC – MS) at the Drug Testing Laboratory, National Administration for Food and Drug, Administration and Control (NAFDAC) Kaduna State, Nigeria and Fourier Transform Infrared Spectroscopy (FT-IR) at the R&D Laboratory, Ahmadu Bello University. Zaria Kaduna State, Nigeria following the method of spectral analyses reported by [21].

Infrared Spectroscopy

The samples for FT-IR analysis were ground up by combining powdered KBr with dry blended powders in a ratio of 1:5 (Sample: KRr) and then compressing the resultant to form dics. The FT-IR spectra were recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm -1 in the absorbance mode for 8 to 128 scans at room temperature. The spectra were measured using a deuterated triglycerinesulphate detector (DTGS) with a specific detectivity of 1×10^9 $cmHz^{1/2}$ W^{-1}.

Gas Chromatography Mass Spectrometry (GC-MS)

A 30 cm column which has an internal diameter of 0.25mm was used during this research. The stationary phase was attached to the interior of the glass capillary, thereby eliminating the need for packing a solid support in the column. The components of the mixture were separated in the column, therefore, reaching the ion trap detector as pure compounds. The compounds were pyrolyzed (450 – 615°C), and the volatile pyrolysis products was thereafter separated on a capillary column (the temperature was programmed to range from 220 – 300°C). The full scan mode ranges from 15 – 35 and also 300 – 650. The compounds were ionized by electron impact (EI) through the utilization of a beam of electrons accelerated to 70 eV traversing a gaseous medium, this energy

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

was employed to generate ions by the removal of electrons, concurrently disrupting certain bonds within the compound.

Diverse populations of ions exhibit varying quantities of internal energy. Certain molecules underwent ionization without undergoing fragmentation, resulting in the formation of a "parent ion." A parent ion, or molecular ion, possesses an equivalent mass in atomic mass units to that of the neutral molecules (with the only distinction being the mass of an electron). This ion represents the peak of the highest mass within the spectrum. Numerous ions that were generated possessed adequate internal energy to undergo fragmentation, yielding a smaller mass ion alongside a neutral counterpart. By employing electrons with consistent energy levels to ionize the compounds, the resultant mass spectra demonstrated high reproducibility, not only within a specific instrument but also across other instruments utilizing 70 eV electron impact ionization. Consequently, a series of mass spectra were produced, enabling the identification of an unknown compound through the process of searching and matching against the generated mass spectra.

RESULTS AND DISCUSSION

Results

Extraction Yield

The solvent used for extraction in this research is n-hexane. This solvent is known to be a non-polar solvent. The main reason n-Hexane is used to extract oil from *Citrullus colocynthis* seeds is that as it is known to be a non-polar solvent, it is very good at isolating non-polar substances like lipids and oils. Because of its selectivity, the oil may be extracted very clean while concentrating on bioactive substances like terpenoids and alkaloids, which support the oil's antibacterial qualities. Diverse solvent polarities would cause polar contaminants to be extracted simultaneously, which would complicate the study and possibly lessen the oil's antibacterial activity.

Furthermore, n-hexane is used due to its volatility, which makes it simple to remove following extraction without affecting the oil's delicate constituents. It is the best option for both industrial and research applications because it provides efficient and economical extraction, particularly for large-scale operations. The selection of n-hexane guarantees a targeted and effective extraction of the lipophilic phytochemicals essential to the biological activity of the oil.

The mass of the oil extract obtained from the extraction and percentage yield of 120 g of sample, is shown in Table 1.

The extraction yield is a critical parameter in any extraction process as it quantifies the efficiency of the method in obtaining the desired compound from the raw material. In the present study, the Soxhlet extraction method was employed to extract oil from Citrullus colocynthis seeds, and the extraction yield was determined to be 33.5ml from an initial seed weight of 120g.The extraction yield of 33.5ml indicate that approximately 33.5g of oil were obtained from the seeds. The yield also served as a reference point for future experiments, ensuring a reasonable degree of reproducibility in the research process and had economic implications for evaluating the cost-effectiveness of large-scale production. A higher yield generally implied a greater concentration of potentially active compounds, which could positively influence the oil's ability to exhibit antimicrobial properties. However, it's important to note that the extraction yield alone didn't guarantee antimicrobial efficacy, and further studies were necessary to evaluate the oil's potential in this regard.

Thin – Layer Chromatography (TLC)

Table 2 gives details of the TLC profile of the oil extract, with n-hexane: petroleum ether (6:4) as the best solvent system for the separation.

Thin Layer Chromatography (TLC) was conducted to analyse the oil extract's composition. The Thin Layer Chromatography (TLC) analysis conducted using n-hexane and petroleum ether as the solvent system provided interesting insights into the separation behaviour of the analysed compounds.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

The result revealed that n-hexane and petroleum ether (6:4) respectively was the most effective solvent system for compound separation. This choice was significant as it provided optimal separation, enabling clear visualization of individual compounds and their relative migration distances (Rf values) on the TLC plate, as documented in Table 1.

Table 1: Result of Thin Layer Chromatography (TLC) Analysis

Solvent	**Yield**	**Colour**	**Sample**	**No. of Spots**	**R_f values**
n-hexane	33.50	Orange	120g	3	0.41, 0.58, 0.63

Phytochemical analysis

The phytochemical screening of the n-hexane oil extract of the seeds of *Citrullus colocynthis*, showed that the extracts of this plant contained alkaloids, saponins, steroids, flavonoids, phenols, tannins, terpenoids and Phlebotomine. (Table 3).

Table 2; Results of the phytochemical screening of the crude oil extracts of the seeds

Phytochemical Compounds	**Present (+) /absent (-)**
Alkaloids	+
Saponins	+
Cardiac Glycosides	_
Cyanogenic Glycosides	_
Flavonoids	+
Steroids	+
Phlebotomine	+
Tannins	+
Terpenoids	+
Terpenes	+
Triterpenoids	_

These phytocompounds occur naturally in plants. Some tannins and flavonoids are responsible for antimicrobial activity by increasing colonic water and electrolyte reabsorption [22]. Similar observations were made on certain Citrullus species [25] about its antimicrobial activity. These phytocompounds possess the ability to initiate human physiological activities such as the stimulation of phagocytic cells and mediation of tumor activities according to [20].

Antibacterial Activities

The result of the antibacterial screening of the oil extract of Citrullus colocynthis against clinical isolates of the microorganisms - Escherichia coli, Salmonella typhi, Staphylococcus aureus and also Pseudomonas aeruginosa is presented in Table 4. The images of the antibacterial activities are shown in figure 3 below.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

Figure 3; Images of antibacterial activities of Citrullus colocynthis Table 3: Result of Antibacterial Activity-

measured in mm

(with Ciprofloxacin as Positive Control)

Conc. (mg/ml)	**Diameter of Zones of Inhibition (mm)**						**Standard error values**	
	E. coli	S. typhi	P. aeruginosa	S. auerus	E. coli (SE)	S. typhi (SE)	P. aeruginosa (SE)	S. aureus (SE)
200	4.0	4.6	5.3	6.5	0.415	0.486	0.540	1.852
400	4.9	6.0	6.8	6.6	0.415	0.486	0.540	1.852
600	4.5	5.9	4.6	6.1	0.415	0.486	0.540	1.852
Control (Ciprofloxacin)	5.7	6.6	5.2	0.00	0.415	0.486	0.540	1.852

Key: +ve = Positive, Positive Control used; Ciprofloxacin antibiotics.

SE = Standard error values calculated based on replicating the experiments three (3) times

E. coli = Escherichia coli, S. typhi = Salmonella typhi, P. aeruginosa = Pseudomonas aeruginosa, S. aureus = Staphylococcus aureus.

From this table, the zone of inhibition of the extracts against the microbes ranges from 4.0 mm to 6.8 mm while that of the control ranges from 5.2 mm to 6.6.mm. The control does not show any zone of inhibition for n-hexane extract for Staphylococcus aureus indicating that the n-hexane extract is more active against these microorganisms than the control.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

In this study, the bacterial activity of Citrullus colocynthis oil extract and Ciprofloxacin (the control used) was assessed against a panel of pathogenic bacteria, including Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, and Staphylococcus aureus. The primary objective was to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of these antimicrobial agents to evaluate their effectiveness against the target organisms.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

The experimental procedure involved the preparation of three different concentrations of Citrullus colocynthis oil extract (200mg/ml, 400mg/ml, and 600mg/ml), as well as a 200mg/ml solution of Ciprofloxacin (antibiotic) as a control. Each concentration was tested independently against the bacteria. Bacterial cultures were inoculated onto agar plates, and each plate featured three wells.

Within these wells, 0.1 mL of Citrullus colocynthis oil extract (alone), Citrullus colocynthis oil extract combined with Ciprofloxacin, and Ciprofloxacin alone (control) were introduced. Following a 24 hours incubation at 26.4°C, the diameter of the zone of inhibition (ZOI) was measured using a standard ruler.

The results revealed that all the tested microorganisms displayed sensitivity to both Citrullus colocynthis oil extract and Ciprofloxacin. Therefore, Staphylococcus aureus and Pseudomonas aeruginosa exhibited larger zones of inhibition compared to Escherichia coli and Salmonella typhi.

The findings prove the potential therapeutic applications in the treatment of bacterial infections, but further research is needed to better understand the efficacy of these antimicrobial agents. In the study, the Citrullus colocynthis oil extract was tested at concentrations of 200 mg/mL, 400 mg/mL, and 600 mg/mL, while Ciprofloxacin, the positive control, was used at a fixed concentration of 200 mg/mL. This means that the lowest concentration of the oil extract (200 mg/mL) was equal to the control. The 400 mg/mL concentration of the extract was twice as high (2-fold) compared to the control, and the highest concentration of 600 mg/mL was three times (3-fold) higher than the Ciprofloxacin control.

Antifungal Activities

Table 5 shows the result of antifungal susceptibility test of the oil extract of Citrullus colocynthis against clinical isolates of Candida albicans

Table 5: Result of Antifungal Activity (with fluconazole as positive control)

Conc. (mg/ml)	**Diameter of Zones of Inhibition (mm)**	**Standard Error value**	**Isolated Components**
	Candida albicans	Candida albicans (SE)	
200	4.0	0.463	+ve
400	4.9	0.463	+ve
600	5.6	0.463	+ve
Control (Fluconazole)	6.0	0.463	

Key; +ve = Positive

SE = Standard error values calculated based on replicating the experiments three (3) times

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

The antifungal properties of the oil extract against Candida albicans, a common pathogenic fungus, were assessed. The primary objective was to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the oil extract, which are crucial in evaluating its efficacy against Candida albicans. The findings could have significant implications in the development of antifungal agents and the potential treatment of Candida-related infections.

The results of this study as shown in Table 6, clearly indicated that the oil extract, at various concentrations, exhibited antifungal activity against Candida albicans. The conclusion that the greatest effect was observed at 600mg/mL was noteworthy. This suggests that at this concentration, the oil extract was able to inhibit the growth of Candida albicans, possibly indicating a lower MIC and it may have reached the MFC, implying that it not

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

only inhibited but also killed the fungus. The oil extract showed comparable inhibition against Candida albicans, when fluconazole was used as a control. The zone of inhibition increased with concentration, but did not reach the potency of fluconazole, which aligns with findings from other plant-based extracts that demonstrate moderate antifungal activity [39].

The determination of MIC is critical because it signifies the lowest concentration of an antimicrobial agent at which visible growth of the microorganism is inhibited. On the other hand, MFC represents the lowest concentration at which the agent is fungicidal, effectively killing the fungus. In this context, achieving a greater effect at 600mg/mL implies that this concentration is highly effective in inhibiting and potentially eradicating Candida albicans.

Comparing with the previous literature, the antimicrobial potential of Citrullus colocynthis is attributed to bioactive compounds like cucurbitacin, which had been widely studied for their pharmacological effects, including antibacterial, antifungal and antiviral activities [14]. Cucurbitacins are tetracyclic triterpenes commonly found in the Cucurbitaceae family, to which Citrullus colocynthis belongs and are known to disrupt bacterial cell walls and inhibit the growth of both gram-negative and gram-positive bacteria.

Table 6; Result of the Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the n-hexane oil extract of the seeds of Citrullus colocynthis against the test microorganisms.

Organisms	**Conc ($x10^2$) $\mu g/cm^3$**	**Colour change**	**MIC ($x10^2$) $\mu g/cm^3$**	**MIC of Ciprofloxacin ($x10^2$ $\mu g/cm^3$)**	**Colony growth**	**MBC ($x10^2$) $\mu g/cm^3$**	**MBC of Ciprofloxacin ($x10^2$ $\mu g/cm^3$)**
E. coli	8	None	-	-	None	-	-
	7	None	-	-	None	7	8
	6	None	6	6	Scanty	-	-
	5	Light pink	-	-	Moderate	-	-
	4	Moderate pink	-	-	Heavy	-	-
Salmonella typhi	8	None	-	-	None	8	8
	7	None	-	-	Scanty	-	-
	6	None	6	6	Moderate	-	-
	5	Light pink	-	-	Heavy	-	-
	4	Moderate pink	-	-	Heavy	-	-
S. aureus	8	None	-	-	None	-	-
	7	None	-	-	None	7	8
	6	None	-	-	Scanty	-	-
	5	None	5	6	Moderate	-	-

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

	4	Light pink	-	-	Heavy	-	-
Pseudomonas aeruginosa	8	None	-	-	None	-	-
	7	None	-	-	None	7	8
	6	None	6	6	Scanty	-	-
	5	Light pink	-	-	Moderate	-	-
	4	Moderate pink	-	-	Heavy	-	-
Candida albicans	8	None	-	-	None	-	-
	7	None	-	-	None	7	8
	6	None	6	6	Scanty	-	-
	5	Light pink	-	-	Moderate	-	-
	4	Moderate pink	-	-	Heavy	-	-

The MIC and MBC values are recorded for each test microorganism and are expressed in µg/cm³. The result provides insight into the antimicrobial activity of the oil extract when compared to the control. This MIC result shows that the oil extract can inhibit the growth of these microorganisms at a lower concentration than the reference standard (Ciprofloxacin). These microorganisms cause diseases such as cholera, typhoid fever, dysentery, diarrhoea, whooping cough and Athlete's foot [20], [39]. The MBC result of this extract also show that this extract can kill these microorganisms at a concentration of 700 to 800 µg/cm^3.

Characterization

The results of the spectral analyses of the compound isolated from the Citrullus colocynthis oil extract is presented in Figure 3 (FTIR) and Figure 4 (GC-MS)

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

FTIR Spectrum of the isolated compound (Appendix I) gave a strong absorption at 2855.1 cm^{-1} which is a characteristic of alkanes (C-H). Absorption at 3011.7 cm^{-1} was also observed for C=C in aromatics. Similarly, broad absorption at 2922.2 cm^{-1} indicates the presence of C-H group corresponding to the absorption at 3420 cm^{-1} of the compound as reported by [29]. Strong absorption at 146.1 cm^{-1} confirmed the presence of carbon to hydrogen double bonds (C=H). Additionally, an absorption at 1707.1 cm^{-1}, which is a characteristic of carbonyl carbon of an ester (C=O). Absorption at 1412.7 cm^{-1} which is due to C-H stretch of alkane, 1278.5 cm^{-1} which is due to presence of (C-N) amines, 1244.9 cm^{-1} which is due to C-O stretch of alcohol, 728.1 cm^{-1} which is due to C-H stretch of alkane, 943.0 cm^{-1} indicate the presence of (C=H) alkene 989.1 cm^{-1} corresponds to aromatic ring, was also observed as reported by [29].

Gas Chromatography Mass Spectrometry (GC – MS) Analysis Identification of Compounds

Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

and Technology (NIST) having more than 62,000 patterns. The spectrum of the known component was compared with the spectrum of the unknown components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

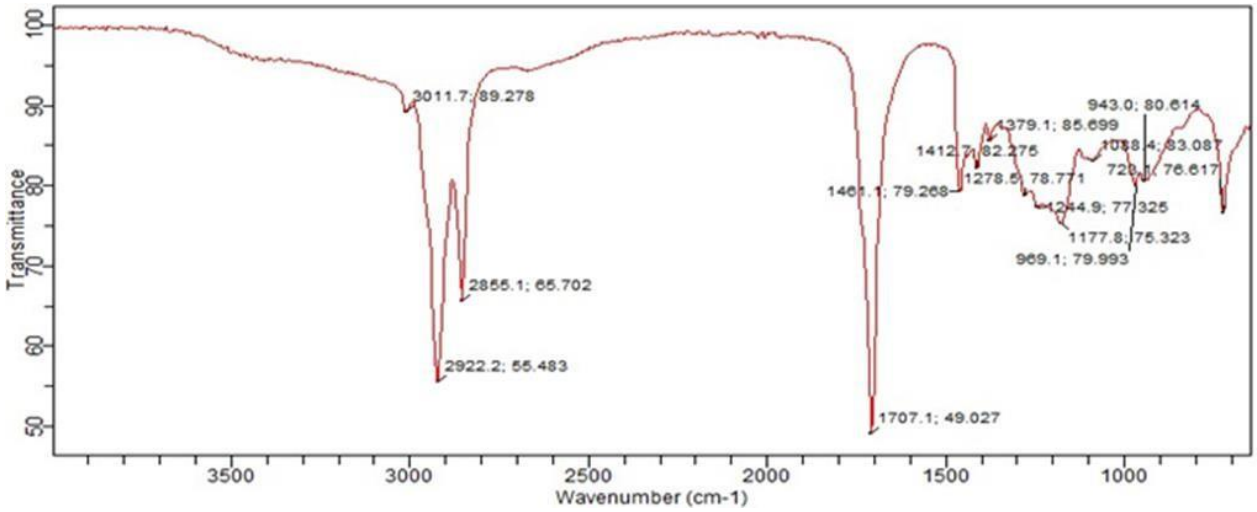
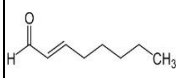
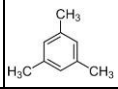
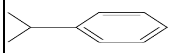
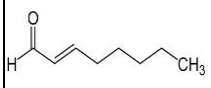
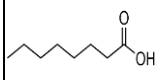
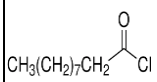
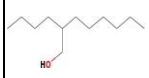
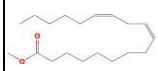
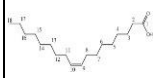
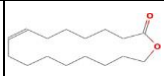
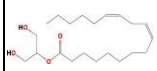
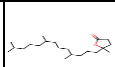
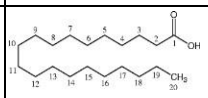
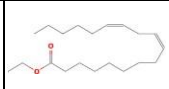
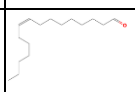


Figure 4: Spectrum showing Citrullus colocynthis FTIR spectra

Table 7: Phytochemical components identified in the oil extract of C. colocynthis

	Retention Time	Compound Name	Molecular Formula	Molecular Weight	Structure	Area (%)
1	3.585	1-Decene	$C_{10}H_{20}$	140.29g/mol		0.05
2	3.625	Benzene, propyl-	$C_6H_5C_3H_7$	120.20g/mol		0.16
3	3.765	ene, 1-ethyl-4- methyl-	C_9H_{12}	120.19g/mol		0.22
4	3.832	Mesitylene	C_9H_{12}	120.19g/mol		0.64
5	3.963	Benzene, (1-methylethyl)-	$C_6H_5C(CH_3)_3$	234.36g/mol		0.09
6	5.055	2-Octenal, (E)-	$C_8H_{16}O$	128.21g/mol		0.07
7	5.625	5-Tetradecene, (E)-	$C_{14}H_{28}$	200.38g/mol		0.11
8	5.732	Octanoic acid	$C_8H_{16}O_2$	144.21 g/mol		0.52
9	6.797	Dodecane	$C_{12}H_{26}$	170.34 g/mol		1.44
10	7.240	Decanoyl chloride	$C_{10}H_{19}ClO$	188.71 g/mol		0.17
11	8.182	adienal, (E, E)-	$C_{10}H_{16}O$	152.24 g/mol		0.14

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

12	8.982	3-Nonen-2-one	$C_9H_{16}O$	140.22 g/mol		0.91
13	9.278	1-Octanol, 2-butyl-	$C_{12}H_{26}O$	186.34 g/mol		0.08
14	9.442	9-Oxononanoic acid	$C_9H_{16}O_3$	172.22 g/mol		0.43
15	11.102	n-Hexadecenoic acid	$C_{16}H_{32}O_2$	256.42 g/mol		0.39
16	17.377	1-Hexadecanol	$C_{16}H_{34}O$	242.44 g/mol		30.61
17	18.875	9,12-Octadecadienoic acid (Z, Z)	$C_{18}H_{32}O_2$	280.46 g/mol		0.09
18	18.967	ecenoic acid, (E)-	$C_{18}H_{34}O_2$	282.47 g/mol		0.17
19	19.671	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48 g/mol		24.50
20	19.879	9,12-Octadecadienoic acid (Z, Z)	$C_{18}H_{32}O_2$	280.46 g/mol		15.35
21	20.064	Oxacycloheptadec-8- en-2-one	$C_{18}H_{32}O_2$	280.46 g/mol		0.97
22	20.318	Octadecadienoic acid (Z, Z)	$C_{18}H_{32}O_2$	280.46 g/mol		0.08
23	20.480	Octadecanoic acid, 2-hydroxy-1	$C_{18}H_{36}O_3$	300.49 g/mol		0.62
24	21.182	Oleic Acid	$C_{18}H_{34}O_2$	282.47 g/mol		0.32
25	21.625	4,8,12,16-Tetramethylheptadeca	$C_{21}H_{42}$	294.56 g/mol		0.33
26	21.721	Eicosanoid acid	$C_{20}H_{40}O_2$	312.53 g/mol		0.09
27	21.856	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308.51 g/mol		0.26
28	22.300	Bicycle [10.1.0] tridec-1-ene	$C_{14}H_{24}$	192.34 g/mol		0.06
29	22.803	cis-9-Hexadecenal	$C_{16}H_{30}O$	238.41 g/mol		0.69
30	22.849	Octadecanoic acid	$C_{18}H_{34}O_2$	282.47 g/mol		0.87

The bioactive phytoconstituents present in the oil extract of Citrullus colocynthis identified by GC-MS analysis.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

Comparison of the mass spectra of the constituents with the NIST library, indicates that the 30 phytoconstituents were characterized and also identified. The active principles (compounds) with their retention time (RT), molecular formula, molecular weight and concentration (%) of that 30 phytoconstituents present in C. colocynthis are presented in Table 6. The GC-MS analysis result reveals the presence of 30 phytoconstituents in the oil extract of C. colocynthis were 1-Decene, Benzene, propyl-, Benzene, 1-ethyl-4-methyl-, Mesitylene, Benzene, (1-methylethyl)-, 2-Octenal, (E)-, 5-Tetradecene, (E)-, Octanoic acid, Dodecane, Decanoyl chloride, 2,4-Decadienal, (E,E)-, 3-Nonen-2-one, 1-Octanol, 2-butyl-, 9-Oxononanoic acid, n-Hexadecenoic acid, 1-Hexadecanol, 9,12-Octadecadienoic acid (Z,Z), 9-Octadecenoic acid, (E)-, Octadecanoic acid, 9,12-Octadecadienoic acid (Z,Z), Oxacycloheptadec-8-en-2-one, 9,12-Octadecadienoic acid (Z,Z), Octadecanoic acid, 2-hydroxy-1, Oleic Acid, 4,8,12,16-Tetramethylheptadeca, Eicosanoid acid, Linoleic acid ethyl ester, Bicyclo[10.1.0]tridec-1-ene, cis-9-Hexadecenal.

The GCMS of compound G1 (Figure 4) gave the molecular weight of the molecule as 41. The signal at 36 corresponds to the loss of CH_4 and signal at 180 corresponds to the loss of COOH. The signal at 167 corresponds to the loss of CH. The signal at 111 is due to the loss of C_4H_8. The signal at 93 due to the loss of water molecule. The signal at 55 corresponds to the loss of C_3H_7. The signal at 29 is due to the loss of C_2H_5. [27]

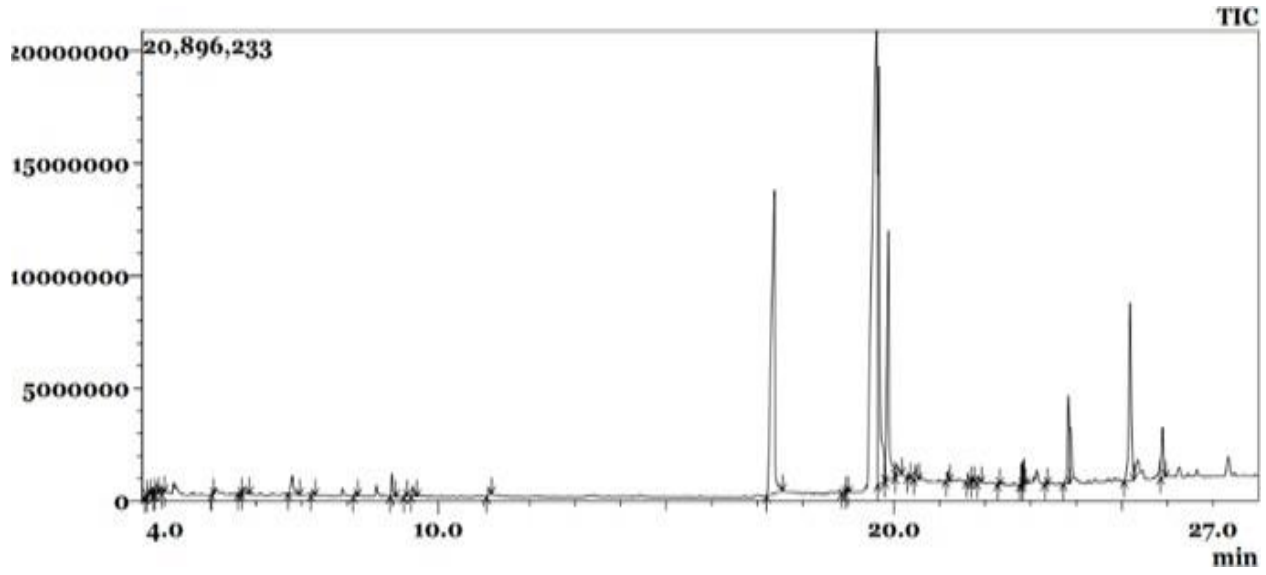


Figure 5: Gas Chromatographic Mass-Spectroscopy (GC-MS) Chromatogram of Citrullus colocynthis

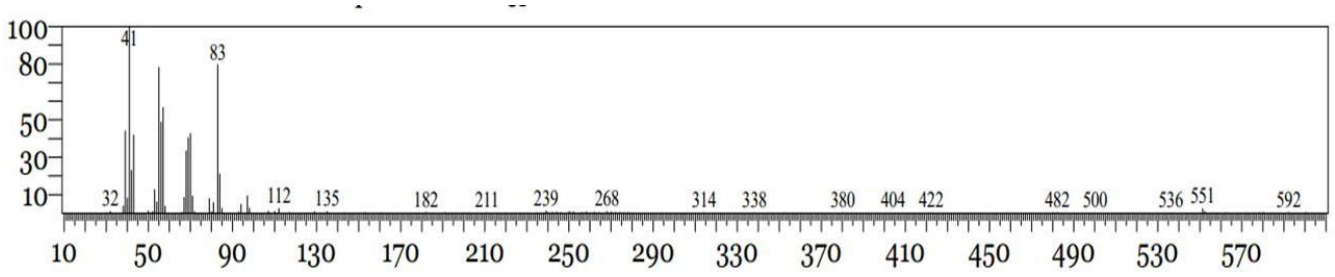


Figure 6: Mass spectra of Citrullus colocynthis

CONCLUSION

This research successfully addressed a comprehensive set of objectives, contributing to a deeper understanding of the oil's potential as an antimicrobial agent. The study began with the extraction of Citrullus colocynthis seed oil and the meticulous preparation of standardized oil samples for antimicrobial testing. This critical initial step laid the groundwork for the subsequent investigations. A diverse panel of microorganisms, including Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, and Candida albicans, was selected for in vitro testing, ensuring a broad assessment of the seed oil's antimicrobial efficacy. The

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

findings revealed that Citrullus colocynthis seed oil exhibited inhibitory effects against these microorganisms, underscoring its antimicrobial potential.

Furthermore, the thin Layer Chromatography (TLC) analysis was conducted to isolate a single compound within the seed oil, shedding light on its chemical composition. The research also involved determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) values of the oil against the tested microorganisms. These efforts provided insights into the seed oil's efficacy and set the stage for potential synergistic effects when combined with standard antibiotics or antifungal agents. Additionally, the research delved into the mechanisms underlying the antimicrobial properties of the seed oil, advancing the understanding of how it exerts its effects.

Finally, this comprehensive investigation not only established the antimicrobial potential of Citrullus colocynthis seed oil but also uncovered valuable information about its mechanisms of action. These findings have far-reaching implications, potentially influencing the development of natural antimicrobial agents with applications in the fields of medicine and public health. Future research may further explore the clinical and therapeutic potential of Citrullus colocynthis seed oil in combatting microbial infections.

DECLARATIONS

Ethics approval and consent to participate

This study involved In vitro experiments on the oil from the seeds of Citrullus colocynthis (plant cells and tissues), no ethical approval from an institutional review board (IRB) or informed consent from participants was required. The plant materials used in this study were obtained from commercial sources and identified at the Herbarium with a deposited voucher number. No endangered or protected plant specie were used. All experiments were conducted in accordance with relevant institutional and national guidelines for plant research. Authors declare that the research complies with the ethical standards of the journal and relevant international norms.

Consent for publication

All authors have reviewed the manuscript and have given their consent for its publication. We confirm that the manuscript has not been published previously and is not under consideration for publication elsewhere.

Availability of Data and materials

The plant materials including the seeds used in this research are available at Ilorin Central Market, Ilorin West Local Government Area of Kwara State, Nigeria upon request. All data and materials used will be made available in accordance with the Journal’s data sharing policy.

Competing interests

The Authors declare that there are no competing interests that could influence the publication of this manuscript.

Funding Declaration

No funding was received for this study from any organization. The authors declare no conflict of interests, financial or otherwise, related to the publication of this manuscript. The Research did not receive any specific funding. We declare that we have no financial or personal relationships that could influence the publication of this manuscript.

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ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue XII December 2024

Author's Contributions

All authors read and approved the manuscript.

Author's Initial	Contributions
Ah	Methodology, investigation, writing-original draft.
Mos	Conceptualization, Designed the study methodology, supervision
Aat	Designed the study, review and editing
Tta	Conceptualization, review and editing
Eo	Collection and identification of seeds, supervision
Am	Provision of equipments used for the Research, Resources, supervision

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