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EVALUATION OF ANTIBACTERIAL PROPERTIES AND GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS) PROFILE OF ESSENTIAL OIL FROM LEMON PEEL

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ABSTRACT

Citrus plants are vital sources of essential oils, both for food and for therapeutic purposes. This study aims to assess the antibacterial properties and Gas chromatography-mass spectroscopy profile of essential oils derived from citrus lemon peel. Lemons were sourced from local vendors, and essential oils were extracted from the peel using steam distillation. Standard methods were employed for qualitative phytochemical analysis, while the anti-microbial activity of the crude oil extract was tested against a clinical isolate of *E. coli*. Chemically, the components of crude oil extract were analyzed using GC-MS. An extraction yield of 8.28 % was achieved. Phytochemical screening indicated the presence of secondary metabolites such as terpenoids, polyphenols, saponins, tannins, and flavonoids. The antimicrobial assay demonstrated that both the crude extract and positive control (metronidazole 200µg/ml) exhibited significant inhibitory activities against *Escherichia coli*. Out of the total essential oil, forty-five (45) chemical components were identified. Limonene having a percentage of 40.07 was the most prevalent constituent. Other constituents included: Gamma Terpinene (11.48 %), β-Pinene (10.11 %), α-Terpineol (8.50 %), α-Pinene (3.80 %), Myrecene (1.57 %), Geraniol (1.50 %), α-Terpinene (1.42 %), α-Terpinolene (1.37 %), Linalool (1.22 %), and cis-α-Bergamotene (1.09 %). The GC-MS analysis highlighted Limonene as the most abundant component in citrus lemon essential oil. This study reveals the potential antibacterial property of essential oils from *C. limon* peel against *E. coli*.

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INTRODUCTION

The most abundant source for drug development leads has been attributed to natural products. Currently, several new drug products are undergoing clinical trials, especially as antibiotics and anti-cancer agents [1,2]. New compounds that are easily produced in microbes as well as from natural products are becoming widely available owing to the application of molecular biological techniques. Additionally, screening libraries that mimic drug-like compounds are being created using combinatorial chemistry techniques and natural product frameworks. The use of natural products in drug discovery has increased, devising screening strategies to enhance data

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mining along with virtual screening techniques on natural product databases. The goal is to make the application of natural products more efficient and effective, thereby improving the drug discovery process [3]. A major benefit of natural products is their availability, cost-effectiveness, and reliability, which they bring to clinical research. Historically, natural products have not only served as medicinal products but have also unveiled new physiological aspects. For instance, digitalis from foxglove highlighted the role of sodium-potassium-ATPase; morphine led to the discovery of receptors affected by endogenous opioids; muscarine, nicotine, and tubocurarine helped in exploring different types of acetylcholine receptors, among others [4]. Although pharmaceutical companies reduced their reliance on natural products for drug discovery at one point, many promising drug candidates of natural origin are currently under development. Recent advancements have reduced the technical hurdles in natural product research, opening up new possibilities to investigate the biological activities of natural product sources that were previously difficult to access. There is an increasing awareness of the chemical diversity inherent in natural products [5]. Essential oils (EOs), a type of natural products, are derived from various parts of plants, including roots, leaves, stems, bark e.t.c. Chemically, they consist of mixtures that encompass a spectrum of fractions, from the most volatile to the heaviest terpenoids. Terpenoids are natural compounds typically produced as secondary metabolites and function as protective chemicals for plants [6]. The scent of terpenoids is often pleasing to humans, and they generally have a lower density than water, with each species exhibiting a unique color that varies in intensity, highlighting their importance in plant species [7]. Due to these attributes and their significant potential applications, the EO market has been expanding rapidly. The aromatic, flavoring, and pharmaceutical properties of EOs are vital across various industry sectors, serving as raw materials for pharmaceuticals, aromatherapy, cosmetics, food flavorings, homecare products, and preservation [8]. Large-scale EO production has been a major factor in the global market; however, high market prices frequently present a substantial challenge. Nevertheless, the strong demand for EOs, especially as natural preservatives, has created growth opportunities in the global market [9].

Citrus plants belong to the Rutaceae family, with several species cultivated in Iran. The primary species include *C. grandis*, *C. medica*, *C. nobilis*, *C. aurantifolia*, *C. aurantium*, *C. Ilimon*, *C. paradise*, and *C. sinensis*. Essential oils, present in varying concentrations, are important natural mixtures composed of diverse chemical compounds [10]. These oils contain compounds that are divided into two categories, each with distinct biosynthetic pathways: terpenoids, which originate from the intermediate acetate-malonic acid, and aromatic compounds, which are formed from shikimic acid and phenylpropanoids. In addition to this classification, essential oil components can be classified by the functional groups present in their structure, including phenols, hydrocarbons, ketones, alcohols, aldehydes, esters, phenolic ethers, oxides, and peroxides. Terpenes and their oxygenated forms, known as terpenoids, are the primary elements of essential oils. Terpenes are further classified based on the number of isoprene units they contain. This classification includes hemiterpenes with 5 isoprene units, monoterpenes with 10 isoprene units, sesquiterpenes with 15 isoprene units, diterpenes with 20 isoprene units, sesterterpenes with 25 isoprene units, triterpenes with 30 isoprene units, tetraterpenes with 40 isoprene units, and polyterpenes (C5) n, where n is greater than 8. Limonene, a monoterpene with a single ring, is present in citrus peel [11]. Despite the pharmaceutical industry's development of numerous new antibiotics over the past thirty years, microorganisms have increasingly developed resistance to these drugs. Bacteria inherently possess the genetic capability to transfer and acquire resistance to conventional antibiotics [12]. The use of plants, which have been a vital source of natural products for enhancing human health over the centuries, as antimicrobial agents, holds significant potential in therapeutic applications. Many plants are utilized for their antimicrobial characteristics, attributed to compounds produced during the plant's secondary metabolism. These products are recognized for their active substances, such as phenolic compounds found in essential oils, as well as tannins. Essential oils are particularly effective in managing biofilm cultures due to their superior infusibility and mode of contact. Consequently, essential oils and other plant extracts have garnered interest as sources of natural products, being evaluated for their potential as alternative treatments for various infectious diseases. Additionally, citrus fruit fiber contains bioactive compounds like polyphenols, with vitamin C (ascorbic acid) being the most notable, which helps prevent and treat vitamin C deficiency, the cause of scurvy. The antimicrobial activity of peel extracts is directly linked to their constituent components [13].

Citrus limon is often consumed as a fruit with its juice used as a characteristic ingredient in many desserts and pastries, including the traditional American lemon meringue pie. Globally, the distinctive astringent flavour of *Citrus limon* has been used to enhance many dishes. The juice of a popular warm-weather beverage, known as lemonade made from lemons is commonly added to tea to improve its taste. Lemon juice contains the B vitamins in smaller amounts such as thiamine, riboflavin and niacin. It has also been reported that lemon juices contain about 5 % citric acid by weight. [14].

Over the centuries, one of the applications of lemon juice has been for the treatment of diarrhea. Individuals of all ages consume lemon juice during episodes of diarrhea, and it is notably the primary cause of death among children and infants [15]. Without proper treatment, diarrhea can result in dehydration, potentially resulting in death. The increased incidence of child mortality due to diarrhea necessitates precise diagnosis [16]. Diarrhea is characterized by liquid bowel movements occurring more than three times daily, sometimes accompanied by blood or mucus. It is a serious illness that can cause both discomfort and death.

Annual reports by Nurfita [16] revealed about 55 % of all diarrhea cases occur during childhood, and its severity is more with children under five. Several factors, such as microbes, malabsorption, allergies, and poisoning, can directly or indirectly cause diarrhea by inducing intestinal inflammation or, in children, through the consumption of contaminated food and drinks [17]. After rotavirus, *Escherichia coli* infection is the second most prevalent cause of diarrhea. *Escherichia coli*, a commensal bacterium, can also act as an intestinal and extraintestinal pathogen, leading to urinary tract infections, meningitis, and septicemia [18]. In developing countries, enterotoxin-producing *Escherichia coli* is a major cause of infectious diarrhea [19]. These pathogens are believed to be responsible for millions of deaths. Although deaths from acute diarrhea have significantly decreased, enteric infections caused by enterotoxin Escherichia coli (ETEC) and other pathogens remain closely linked to factors including: malnutrition, stunted growth, and impaired cognitive development [20]. Many factors have been reported to lead to antimicrobial resistance, which the misuse of antibiotics is a lead. Natural product medicines consisting of natural ingredients are considered safer in comparison with modern chemical drugs as a result of their relatively minor side effects as they are used appropriately [21]. Lemon fruit is one such natural plant, containing medicinal compounds that serve as anticancer, antibacterial, antifungal, antiviral, and antidiabetic agents. Saponin, an alkaloid compound, is responsible for its antibacterial properties [22].

The treatment of infectious diseases is greatly challenged by the rise and spread of antibiotic resistance among bacterial pathogens, especially *E. coli* [23]. The inappropriate use of antibiotics has led to the emergence of multidrug-resistant strains, diminishing the effectiveness of these drugs. Consequently, there is an urgent need to explore alternative methods to combat these infectious agents, particularly those that are cost-effective, accessible, and environmentally sustainable. Research into plant-based products of natural origin is a promising area in the search for alternative treatments for infectious diseases. Scientific investigations aim to validate the claims of traditional medical systems regarding the therapeutic benefits of plant-derived compounds [24]. The *C. limon* (Lemon) has drawn attention among the numerous plant species with possible antimicrobial capabilities because of its wide range of bioactive components, including essential oils [25]. Although, the antibacterial activity of lemon as a fruit has been examined by various researchers, the activity of its bark housing the essential oil against diarrhea implicated *E. coli* and the chemical components present has not been evaluated, hence; this study aimed to determine antibacterial activity and Gas chromatography mass spectroscopy analysis of essential oils of citrus lemon peel.

MATERIALS AND METHODS

Lemon fruits, sterile blade, petri-dishes, distillation unit, measuring cylinder, separating funnel, GC-MS equipment, thermometer, H_2O_2, citrate medium, plasma, Kovac's reagent

Sample Collection

Lemons were sourced randomly from local vendors in Nsukka market, Southeastern Nigeria, as shown in Figure 1.

Figure 1: Whole lemon samples and lemon peels

Preparation Of Lemon Peels

The outer surface of the lemons was peeled after cleaning, followed by the separation of the pith from the colored part of the peels. This is done to allow maximum extraction of oil from the oil sac as most oil is found within the pitch. Pre-heating at a temperature of 45 ^{o}C was allowed and kept for 2 h.

Oil Extraction Via Steam Distillation

The distillation apparatus was set up consisting of the distillation flask that contains the lemon peel, and basket heater which supplies heat to the sample and horizontal condenser (this cool the vapors of the component of the mixture of the lemon peel allowing them to condense to liquid form and be collected through a conical flask.

In a distillation flask, 100 g of lemon peel was combined with 200 ml of distilled water. The distillation unit was heated using a temperature-controlled basket heater. Initially, the experiment was conducted at 88 °C for 60 minutes. The steam produced caused the oil glands to burst, and as it passed through the condenser, the resulting condensate was received in a conical flask. This mixture separated into two layers: oil and water, with the oil floating on top due to its lower density. The oil was easily separated using a separating funnel, labeled, and stored in glass bottles as depicted below. To identify the optimal distillation temperature and duration, the experiments proceeded in several stages. Firstly, the distillation time and solid-to-solvent ratio were kept constant while the temperature was adjusted in 20 °C increase, ranging from 88 °C to 98 °C, to find the optimal distillation temperature. In the next stage, the temperature and solid-to-solvent ratio were kept constant, while the distillation time was altered in 20-min intervals (from 20 to 40 minutes) [26], which helped to determine the optimal distillation time. The experiment involved keeping the distillation time and temperature constant while changing the solid-to-solvent ratio from 100 g/100 ml to 100 g/200 ml, which established the optimal conditions for extracting citrus oil from lemon peels through distillation. The lemon oil was then stored as depicted in figure 2 for further examination.

Figure 2: Extracted Lemon Oil

Qualitative Phytochemical Analysis

Standard protocols were followed to conduct qualitative phytochemical screening of the lemon essential oil to identify the secondary metabolites present [27]. The following tests were carried out:

Test for Terpenoids: A 0.5 ml of oil was mixed with 2 ml of chloroform and 3 ml of sulfuric acid. A resultant reddish-brown color indicates the presence of terpenoids.

Test for Alkaloids: About 2 ml of the oil was dissolved in dilute HCl, followed by the addition of 6 ml of Mayer reagent to 1 ml of the mixture. The formation of a cream-colored precipitate indicates the presence of alkaloids.

Test for Phenols: About 1 ml of the oil extract was combined with three drops of $FeCl_2$ and 1 ml of KFe (CN). A resultant greenish-blue color confirms the presence of phenols.

Test for Tannins: Approximately 2 ml of the oil was boiled with 2.5 ml of distilled water, and 0.1% Ferric chloride was added to the mixture. A blue-black coloration indicates the presence of tannins.

Test for Saponins: Approximately 0.5 ml of oil was mixed with 5 ml of distilled water and agitated. The formation of foam confirms the presence of saponins.

Test for Flavonoids: A few drops of 1 % NH3 solution were added to the lemon oil in a test tube. The presence of flavonoids is confirmed by the appearance of a yellow coloration.

Identification And Characterization of Bacterial Isolate Used

The bacterial isolate, *Escherichia coli,* was sourced from the Pharmaceutical Microbiology & Biotechnology laboratory at the University of Nigeria, Nsukka. The colonies were initially identified based on their Gram character, morphological, and cultural characteristics. Distinct colonies were sub cultured onto freshly prepared media petri-dishes. Biochemical test such as catalase test, coagulase test, Simmon's citrate utilization test, indole, and Voges-Proskauer test were carried out according to standard methods to confirm the bacterial isolates [28]. The bacterial isolates were stored in the refrigerator after further subculturing.

Preparation of McFarland Standards

The McFarland standard, equivalent to approximately 1.5 × 10^8 CFU/ml of bacterial suspension, was prepared and stored in an airtight amber container at 25 °C. The absorbance of 0.5 McFarland Standard was also determined to be approximately 0.13.

Antibacterial Evaluation

The agar well diffusion method was used to conduct the antibacterial evaluation [29]. The samples were labelled as follows: A (lemon oil**),** B (metronidazole 200 µg/mL, positive control), and C (distilled water, negative control**).** Molten and cooled Mueller-Hinton agar (MHA) media were prepared, and about 20 mL was poured into sterilized Petri dishes and allowed to solidify. The test organism was cultured for 24h in MHA broth. Lawns of the test organism (1 × 10^8 CFU/mL) were prepared using a 100 mL broth culture. A sterilized stainless steel cork borer was used to create agar wells of 6 mm diameter, and 6 µL of the A

(lemon oil), B (metronidazole 200 µg/mL), and C (water) were introduced into each well. The plates were examined and measured for zones of inhibition. Each organism's mean value was recorded and expressed in millimeters. The experiment was conducted in triplicate.

GC-MS ANALYSIS AND COMPOUND IDENTIFICATION.

The Shimadzu Training Centre for analytical instruments (STC) in Lagos conducted the GC-MS analysis. The lemon oil was examined using gas chromatography-mass spectrometry with electron ionization (GC-MS/EI). The GC-MS/EI system used is a Scion 436-GC Bruker model paired with a triple quadrupole mass spectrophotometer with fused silica capillary column BR-5MS (5 % Diphenyl/95 % Dimethyl polysiloxane) and Length: 30m, Internal diameter: 0.25 mm, Thickness 0.25 *u*m. Helium gas (99.999 %) was used as the carrier gas at a constant flow rate 3.22 ml/min and an injection volume of 8 ui was employed (split ratio of 5:1). The injector temperature (isothermal for 2 min), with an increase of 12 ^{o}C/2 mins to 240 ^{o}C, then 12 ^{o}C/2 min to 290 ^{o}C. This last increase was to clean the column from any residues [30]. The mass spectrometer was operated in the positive electron ionization (EI) mode with ionization energy of 70 eV. the solvent delay was 0-3.5 min. A scan interval of 0.5 sec and fragments from m/z 45 to 700 Da was programmed. The inlet temperature was set at 250 ^{o}C. The relative percentage. amount of each component was calculated by comparing it`s average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was MS Work station 8. The NIST Version 2.0 library data base of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for identifying the chemical components.

Statistical Analysis.

Descriptive statistical analysis was performed using the basic Microsoft Office Excel functions. The mean of the Inhibition Zone Diameters (IZDs) was recorded as the mean ± standard error of mean

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RESULTS

Percentage Yield

The percentage yield of the extracted oil was calculated below.

$$\text{Percentage yield} = \frac{\text{Amount of citrus oil extracted x 100}}{\text{Total amount of grated citrus peels}}$$

Amount of citrus oil extracted = 4.97 ml, Total amount of grated citrus peels = 600 g

Percentage yield = 8.28 %

Effect of Temperature on Oil Extraction

The result of steam distillation by changing the temperature at an interval of 20 ^{o}C from 88 ^{o}C to 98 ^{o}C while the other parameters (time and solid to solvent ratio) were kept constant was recorded. It was observed in Figure 3 that increase in distillation temperature increased the yield and it became constant at 200 ^{o}C where further increase in distillation temperature had no effect on the yield, which is because at higher temperature, charring of lemon peels takes place at the bottom of the flask.

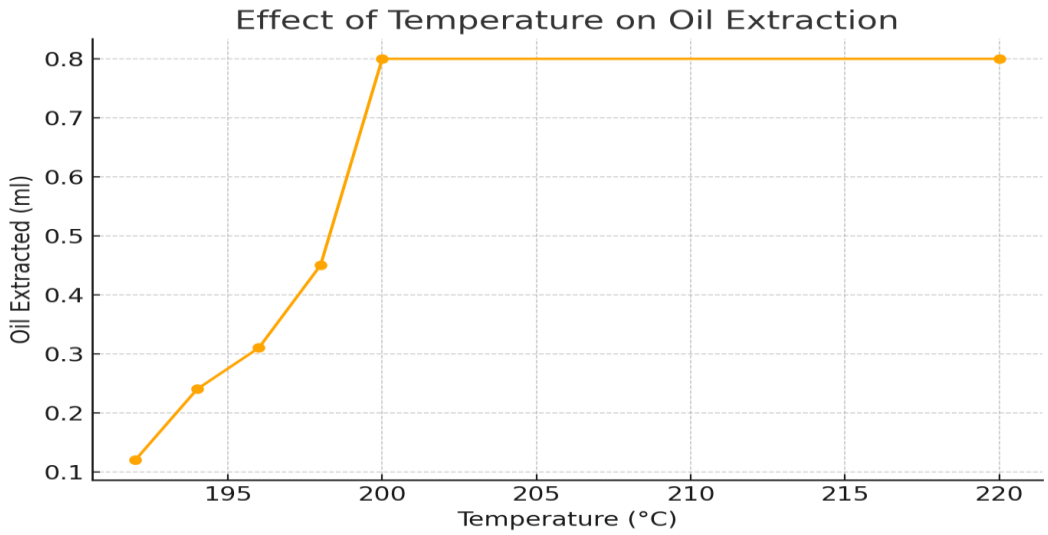

Figure 3: Effect of temperature on oil extraction

Effect of Time during on oil extraction

Figure 4 shows the result of the effect of time on steam distillation from 15 mins to 75 mins, while the other parameters (temperature and solid to solvent ratio) were kept constant. It was also observed in that with increase in distillation time, the yield increased and it is maximum at 60 mins duration.

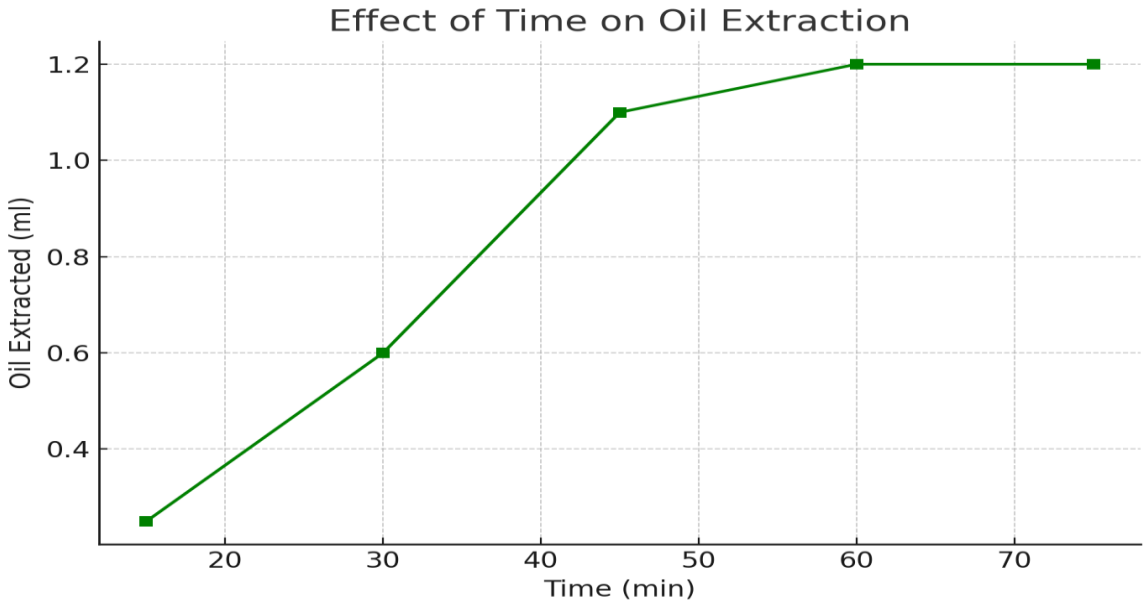

Figure 4: Effect of Time on Oil Extraction

Effect of Solid to Solvent ratio on oil extraction

Figure 5 shows the effect of solid to solvent ratio on steam distillation by changing the solid to solvent ratio from 100

g/120 ml to 100 g/220 ml, while the other parameters were kept constant. It was observed that with an increase in the distillation solid to solvent ratio, the oil yield increased, and it is maximum at a solid to solvent ratio of 100 g/200 ml.

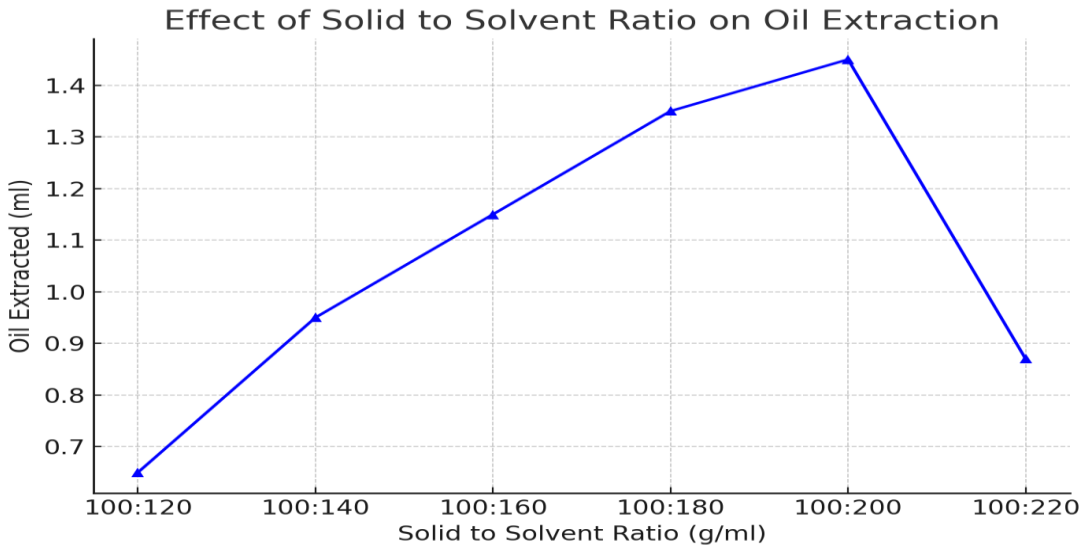

Figure 5: Effect of solid to solvent ratio on oil extraction

Qualitative Phytochemical analysis of *Citrus Limon* Peel Extract

Phytochemical screening of *Citrus limon* peel extract showed positive result for five phytochemicals which includes: Flavonoid, polyphenols, saponins, tannins, terpenoids. Only the alkaloid showed a negative result.

Table 1: Phytochemical screening of *Citrus Limon* peel extracts

S/N	TEST	RESULT
1	Flavonoid	+
2	Polyphenols	+
3	Saponin	+
4	Tannins	+
5	Terpenoids	+
6	Alkaloid	_

(+) Indicate Present, (-) Indicate Negative

Biochemical test

The morphological and biochemical test confirmed the isolate to be probably *E. coli*. Morphologically, the bacterial isolate on EMB showed the appearance of greenish metallic sheen colour. Table 2 recorded positive for catalase, indole, mixed acid fermentation, and glucose utilization and recorded negative results for H_2S,

oxidase, Voges-Prosteur, coagulase, and citrate.

Antimicrobial assay of *C. limon peel* oil

The result of the antimicrobial assay showing the bar charts representing the inhibition zone diameter (IZD) of *Citrus limon* peel crude oil, compared with a positive control (Metronidazole 200 µg/ml) and a negative control (Water). The antimicrobial activity is reported in Table 3 and illustrated in Figure 6.

Table 2: Biochemical Screening

Bacteria	Catalase	H2S	Motility	Indole	Oxidase	Mixed acid Fermentation	Voges Prosteur	Coagulase	Citrate	Glucose utilization
E. coli	+	-	+	+	-	+	-	-	-	+

+ = positive, - = negative

Table 3: Antimicrobial assay of Citrus lemon peel oil

Treatment Code	Treatment	IZD (mm)			Mean IZD ± SEM (mm)
A	Crude oil extract	9	8	9	8.67 ± 0.3333
B	Metronidazole 200 µg/ml	15	14	16	15 ± 0.5774
C	Water	0	0	0	0.0 ± 0.0

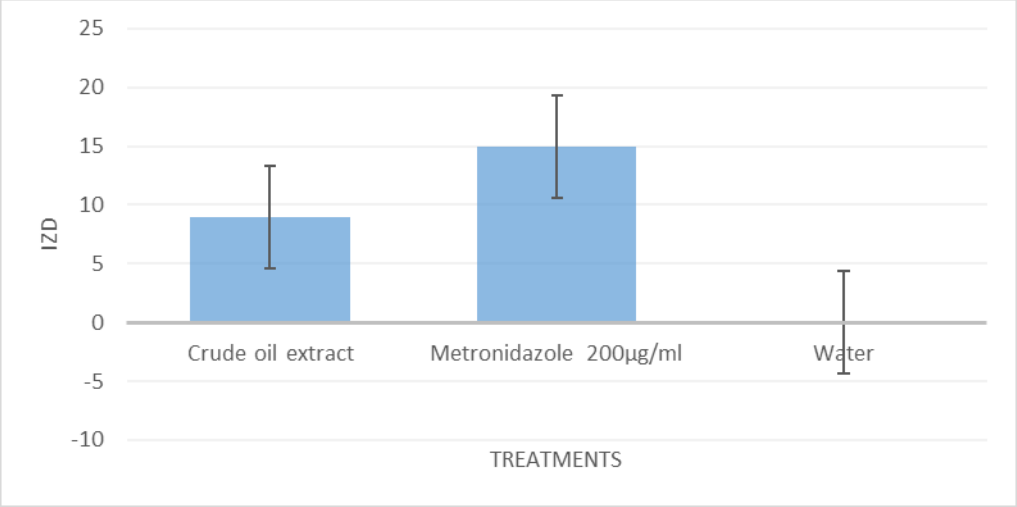

Figure 6: Antimicrobial assay of citrus lemon peel oil

Chromatogram of the GC-MS analysis of Citrus Lemon Oil and compounds identified

The characteristics of citrus oil extracted from Lemon peels are determined by standard method of Gas Chromatograph Mass spectroscope (GC-MS) and the compositions of limonene was found to be (40.07 %) followed by β-pinene (10.11 %), gamma terpinene (11.48 %), α- terpineol (8.50 %), α-pinene (3.60 %), myrecene (1.57 %), geraniol (1.50 %), α-terpinene (1.42 %), α-terpinolene (1.37 %), linalool (1.22 %) and cis-α-bergamotene (1.09 %).

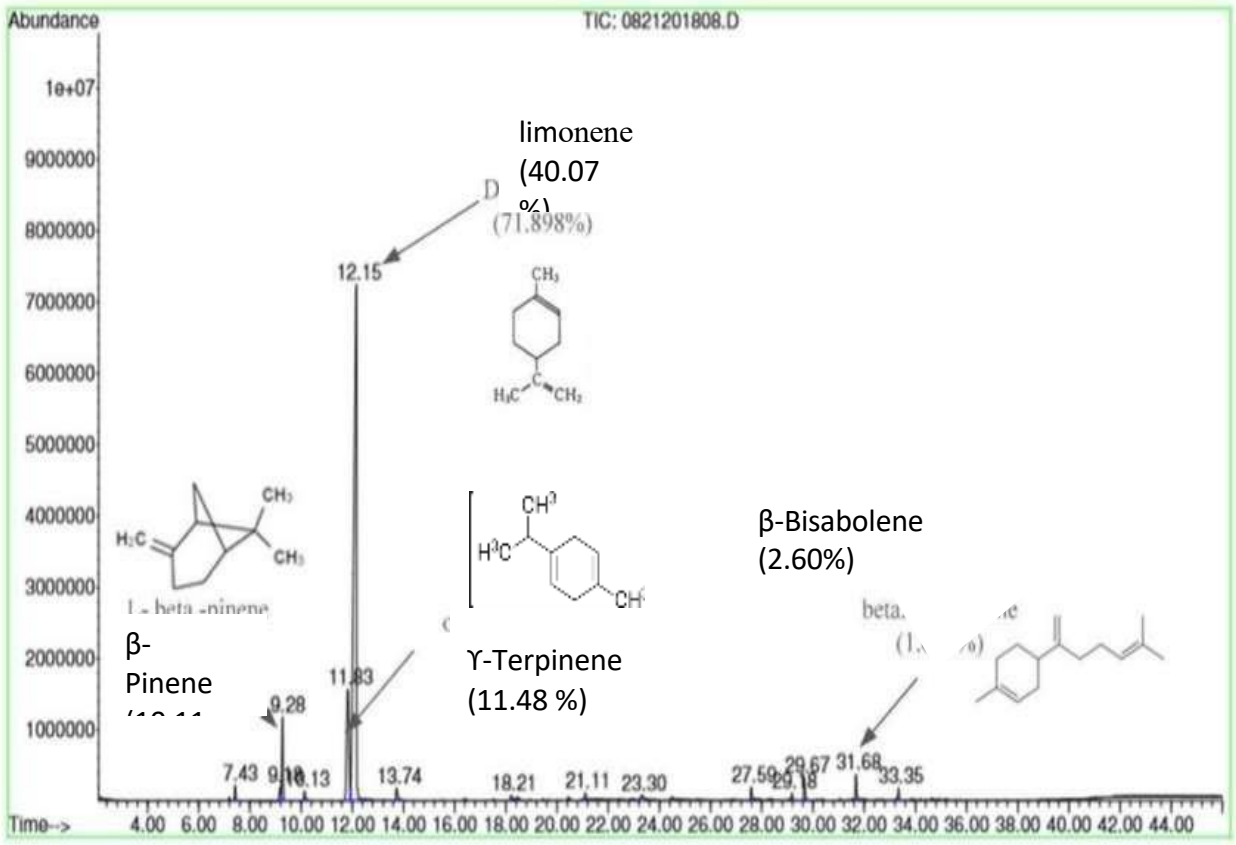

Figure 7: Chromatogram of the GC-MS analysis of Citrus lemon Oil

Table 4: Percentage of forty-five compounds identified from the citrus lemon essential oil after GC-MS analysis.

No	COMOPUNDS	RT (mins)	RETENTION INDEX	AREA (%)
1	α-Thujene	4.254	925	0.17
2	α-Pinene	4.733	932	3.60
3	Camphene	5.199	946	1.48
4	β-Pinene	6.247	977	10.11
5	Myrcene	6.726	991	1.57
6	α-Phellandrene	7.210	1005	0.44
7	α-Terpinene	7.702	1016	1.42
8	Limonene	8.463	1034	40.07
9	Trans-β-ocimene	8.652	1039	0.22
10	*Cis*-β-ocimene	9.054	1048	0.48
11	ϒ-Terpinene	9.582	1061	1.42
12	α-Terpinolene	10.737	1088	1.37
13	Linalool	11.296	1061	1.22
14	Fenchyl alcohol	11.832	1088	0.24
15	Cis-Sabienene hydrate	12.199	1101	0.16
16	β-Ocimene	12.765	1113	0.05
17	β Terpineol	13.231	1121	0.07
18	α-Phellandren-8-ol	14.275	1134	0.35
19	Terpinen-4-ol	14.735	1144	2.25
20	α-Terpineol	15.468	1168	8.50
21	Nerol	17.070	1178	0.25
22	*Cis*-Citral	17.617	1194	0.69
23	Geraniol	18.284	1230	1..50
24	Geranial	18.979	1242	1.45
25	ẟ-Elemene	21.755	1257	0.05
26	Neryl acetate	23.038	1273	0.14
27	Geranyl acetate	23.836	1366	0.22

28	β-Elemene	24.095	1385	0.15
29	*Trans*-Caryphyllene	25.193	1391	0.47
30	*Cis*-α-Bergamtene	25.932	1417	1.09
31	Selina-4,11-diene	27.518	1435	0.16
32	Germacrene D	27.723	1474	0.16
33	β-Caryophyllene	27.911	1479	0.12
34	β-Cadiene	28.165	1484	0.08
35	α-Seliene	28.295	1490	0.08
36	*Cis*-α-Bisabolene	28.680	1501	0.09
37	β-Bisabolene	28.930	1505	2.60
38	ϒ-Maaliene	30..020	1519	0.09
39	Germacrene B	30.720	1528	1.24
40	Cedrol	32.373	1550	0.19
41	Viridiflorol	33.389	1564	0.07
42	Intermedeol	34.459	1578	0.06
43	Caryophyene oxide	35.036	1586	0.09
44	Caryophyene oxide	36.234	1593	0.12
45	Juniper camphor	36.635	1598	0.25

RT- Retention Time, RI- Retention Index

DISCUSSIONS

The result for the effect of temperature on steam distillation during extraction (Figure 3) demonstrated a direct correlation between temperature and the yield of essential oil from citrus lemon peel, up to a certain limit. As the temperature increased from 192°C to 200°C, there was a significant increase in the volume of extracted oil, indicating improved volatilization and release of oil constituents. However, at 200°C and 220°C, the yield stabilized at 0.80 ml, indicating that further temperature increases beyond this point offer no additional yield benefit. This stabilization could be due to the charring of lemon peels at the flask's bottom at higher temperatures.

This result is consistent with findings by other researchers who observed that increasing the steam temperature during the distillation of *Citrus sinensis* peels significantly increased oil yield up to around 200 °C, beyond which degradation of thermolabile compounds or saturation of extraction efficiency occurred [31]. Similar to other studies, it has been revealed that steam temperatures exceeding 200°C during the distillation of *Citrus aurantium* did not further increase yield, and even reduced oil quality was noted [32].

Key findings revealed that beyond 200 °C, both oil yield and quality diminished as key essential volatile compounds such as limonene undergoes thermal degradation. A study highlighted that the diffusivity of essential oils from plant matrices rises with temperature, enhancing oil recovery [33]. The stabilization observed at 200–220°C in this study suggests a thermodynamic equilibrium point, where the rate of oil release equals the rate of volatilization and recovery, making further temperature increases energy-inefficient and potentially damaging to oil composition. Similar studies have shown that increasing distillation temperature, time, and the solid-to-solvent ratio simultaneously boosts oil yield [34,35].

The result of the effect of time on extraction (figure 4) revealed a positive correlation between distillation time and oil yield up to a certain point. At 15 min, the oil yield was 0.25 ml, indicating early-stage volatilization of essential oil compounds and it progressively increased to 0.60 ml at 30 min, 1.10 ml at 45 min, and peaked of 1.20 ml at 60 min, which was maintained through 75 min. Beyond 60 mins, the plateau was observed, suggesting that most volatile components were fully extracted by that time, and prolonged distillation yielded no additional oil. It also noted that 60 min is an efficient and cost-effective duration for maximizing yield while preserving essential oil quality in citrus peels. This finding aligns with the study of Suhail et al. [36], which reported a significant increase in oil yield during the steam extraction of *Citrus limon* peel up to 60 min, after which the yield stabilized. The stabilization was attributed to the exhaustion of readily extractable volatile constituents, and they emphasized that prolonged heating may lead to degradation of sensitive compounds without increasing quantity. Similarly, a study reported that 50–70 min was the ideal duration for extracting essential oils from *Citrus sinensis* peels using steam distillation, with the majority of the oil being collected in the first hour. After this duration, energy input increased without a significant rise in yield, a finding that aligns with our findings [37]. A similar study highlighted that the initial distillation phase recovers the most volatile compounds, such as monoterpenes like limonene, which is the main component in citrus peels. Extending the distillation time might enhance the extraction of less volatile sesquiterpenes, but their overall contribution is minor and could lead to unnecessary thermal exposure and energy use [38].

The impact of the solid-to-solvent ratio on steam distillation efficiency was significant (figure 5), as the oil yield rose steadily from 0.65 ml at a ratio of 100:120 (g/ml) to a maximum of 1.45 ml at 100:200. However, when the ratio was further increased to 100:220, the yield dropped to 0.87 ml, indicating a threshold beyond which efficiency decreases. This underscores the necessity of optimizing the solid-to-solvent ratio for optimal oil recovery. It has also been observed that increasing the solid-to-solvent ratio in steam distillation enhanced essential oil yield up to a certain point, after which the yield diminished. The researchers noted that an excessively high solvent volume could dilute energy transfer or cause excessive condensation, leading to poor

oil recovery [39]. The peak yield at a ratio of 100:200 g/ml represents the ideal mass transfer conditions in this study, where adequate water vapor interacts with the plant material to effectively volatilize and transport oil components. Beyond this optimal point, a higher solvent volume might lower the temperature gradient and extend heating time, which can reduce extraction efficiency or cause premature condensation of the oil before collection.

Additionally, it was observed that lower biomass-to-water ratios resulted in incomplete oil extraction due to insufficient contact between steam and plant material, while excessively high volumes led to reduced extraction temperatures and longer distillation times, negatively impacting oil quality and quantity [40]. Another study on *Citrus sinensis* peel oil extraction reported that the optimal solid-to-liquid ratio was around 1:2 [40], which aligns well with the peak observed at 100:200 (approximately 1:2) in this study.

In table 1, the lemon peel extract revealed maximum phytochemical screening with the presence of flavonoid, polyphenols, terpenoids, saponin, tannins, flavonoid, and tannins as the dominant group of bioactive compounds acting as primary anti-oxidants or free radical scavengers [41]. Alkaloids were shown to be absent. The presence of tannin and phenolic compound specified that the *Citrus limon* peels had potential antimicrobial properties. According to several other studies, various terpenes have proved to show its antimicrobial activities [42]. Polyphenols constitute one of the largest groups of plant secondary metabolites known to harbor numerous vital biological functions such as anti-inflammatory, anti-cancer, anti-microbial and antioxidant properties [42, 43]. Therefore, the presence of polyphenols supports the anti-microbial activities of essential oils extracted from lemon peels. The result of biochemical test in table 2 revealed positive reactions for catalase, motility, indole production, mixed acid fermentation, and glucose utilization with negative reactions for H_2S production, oxidase, Voges-Proskauer, coagulase, and citrate utilization. These biochemical tests were conducted to confirm that the isolate was *E coli.* The result of the biochemical tests aligns with standard results for *E. coli* confirmation [28].

The antimicrobial assay was conducted to evaluate the ability of various treatments to inhibit the growth of *E. coli* (Table 3 and Figure 6). The crude oil extract showed an inhibition zone diameter (IZD) of approximately 10 mm. The presence of a measurable inhibition zone indicates clear antimicrobial activity against *E. coli*. Its effect in comparison with the positive control indicated a significant inhibitory effect. The error bar suggests moderate variability, but the inhibition remains statistically significant compared to water. Metronidazole, which was used as the positive control, exhibited a higher IZD of approximately 15 mm. Accordingly, metronidazole, which is a known antimicrobial agent, displayed stronger inhibitory activity. Its relatively larger error bar suggests some variability, but it is more potent than the crude extract. Water (negative control) showed no measurable inhibition zone (IZD), confirming that the antimicrobial effect is not due to solvent interference. The slight error bar might reflect a measurement artifact but does not indicate any real antimicrobial effect.

The result strongly suggests that *C. limon* peel essential oil possesses antimicrobial properties, though not as potent as the standard metronidazole. This antimicrobial effect is likely due to bioactive constituents such as limonene, and other monoterpenes, which are known to disrupt bacterial cell membranes and metabolic pathways. Lemon extracts, including juice and peel extracts, have demonstrated antibacterial activity against a wide range of pathogenic bacteria, including *E. coli* [3, 44]. This finding aligns with previous studies showing strong antimicrobial activity of *Citrus limon* peel oil, particularly against *E. coli*, *S. aureus*, and *P. aeruginosa*. These studies reported significant antimicrobial activity of citrus essential oils against Gram-positive and Gram-negative bacteria [45]. Another study by Ali et al reported that lemon peel oil exhibited broad-spectrum antibacterial activity, with inhibition zones between 10–18 mm, comparable to standard antibiotics [46].

The result of the GC/MS analysis in Table 4 and Figure 7 revealed a total of 45 chemical compounds. The identified compounds limonene (40.07 %), β-pinene (10.11 %), and gamma terpinene (11.48 %), were the major components while α-terpineol (8.50 %), was in intermediate concentration. The compounds α-pinene (3.80 %), myrecene (1.57 %), geraniol (1.50%), α-terpinene (1.42 %), α-terpinolene (1.37 %), linalool (1.22 %) and cis-α-bergamotene (1.09 %) were present in minor concentration.

Reports available on the chemical composition of *C. limon* abound. One of the reports by Kakoos et al found limonene (29.52 %) as major compounds in peels of C. limon [47]. Another report by Hong *et al* (2017) revealed limonene (31.33 %), γ-terpinene (13.70 %) and βpinene (8.14 %) as major compounds [48].

Studies that reported the presence of limonene as major compound present in essential oil peels of *C. limon* with their percentage abundance includes 64.1-71.1 %, 61.8 - 73.8 %, 57.71 %, 54.60 %, 67.1 % [19, 49-52]. The result of this study (figure 7) also aligns with these researches. Limonene, the major compound present in this study, is a cyclic monoterpene having a lemon like odor and is considered to be the major chemical constituents in various oils of citrus species. The name limonene itself comes from peel of *C. limon*. Limonene is acquired commercially from citrus fruits by applying two primary methods; centrifugation method or steam distillation. As a result of its inherent pleasant aroma, it is used in various perfumery industries and also in enhancing the flavor of various food items. It has also found its application in cosmetic industries and it is regarded as less or no toxicity to the cells of the body. Limonene has wide range of applications as dietary supplement, fragrance ingredient for cosmetic products, in food manufacturing industries, in some medicines, bath products, personal care products, botanical insecticide, cleaning products, solvents, paint and in air fresheners [53-55]. Bertuzzi et al reported the antimicrobial, and antioxidant properties of essential oil from *C. limon* peel [51]. Therefore *C. limon* peel essential oil can be a potential herbal formulation of cost effective, easily available antimicrobial agent. It is a good source for the isolation of pure compound like limonene, β-pinene, gamma terpinene which has an immense commercial and medicinal applications. However, some of the earlier studies revealed few variations in the amount of limonene. It is quite acceptable as the chemical composition of essential oil varies depending on the method of extraction, environmental factors, genetic factors and different geographical location.

CONCLUSION

The antibacterial effect of *C. limon* essential oil against *E. coli*, have shown to have promising result from this study. The study emphasizes the peel of C. *limon* and its essential oil as a potential antimicrobial agent. This study contributes to the search for plant-based antimicrobial agents as alternatives to conventional antibiotics, given the increasing rise of antimicrobial resistance (AMR). Identifying bioactive compounds in lemon peel essential oil could serve as a first investigation for the development of novel antibacterial formulations. Lemon peel, often discarded as waste by both the consumers and in the food and juice industries, is highlighted in this research as a potential cost-effective and sustainable source of antimicrobial compounds, aligning with circular economy and waste valorization strategies. The bioactivity of the essential oil suggests its potential application in topical antibacterial creams, oral hygiene products, and natural preservatives in the pharmaceutical and cosmetic industries. This work establishes a foundation for future studies on structure-activity relationships, synergistic effects with existing antibiotics, and in vivo efficacy assessments. It also encourages the exploration of other citrus peels and plant-derived oils. Given the global concern over multidrug-resistant pathogens, especially *E. coli*, identifying safe, accessible, and effective natural antimicrobials could have significant implications for managing infections, especially in resource-limited settings. Additional research may focus on the precise phytochemicals that cause these effects.

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AUTHORS' CONTRIBUTIONS

Chinenye Nnenna Ugwu – Conceptualization, literature screening, conducting laboratory research, manuscript drafting, funding acquisition, language editing and reference management. **koro Blessing Ezinwanne** – data extraction, visualization, formatting and layout, language editing and reference management. **Okechukwu Nnaemeka Victor** - literature screening, data extraction, and manuscript

drafting. **Anthony Amaechi Attama** – Revising and editing, supervision and validation.

CONFLICTS OF INTEREST

All authors have approved the final manuscript and all declare they have no conflict of interest.

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