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FROM WASTE TO RESOURCE: VALORIZING *CITRUS LIMON* LEAVES THROUGH ESSENTIAL OIL EXTRACTION AND EVALUATION OF ITS ANTIMICROBIAL PROPERTIES

Madiha Batool[a], Uzman Khan[a], Razia Batool[a], Rimsha Afzaal[b], Khizar Hayat[c], Ahmad Ali Khan[d], Mohammad Tariq Qureshi[e], Mahnoor Fatima[f], Shamoon Tariq[g], Samahir Khalid[h], Zartashia Ijaz[i]

[a] Government College University, Lahore

[b] Government Islamia college, civil lines, Lahore

[c] Quaid-i-Azam University Islamabad

[d] North China University of Water Resources and Electric Power Zhengzhou, Henan, China

[e] Government College University Faisalabad

[f] University of Management & Technology, Lahore

[g] Mohammad Ali Jinnah university

[h] University of Engineering and technology Lahore

[i] University of Sargodha

[1] Corresponding Author: Dr. Uzman Khan, E-mail: *uzmanuk@gmail.com*

Tel: (+92)-305-6505302

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ABSTRACT:

Citrus limon (lemon), a member of the Rutaceae family, is renowned for its nutrient-rich leaves, which yield essential oil with notable medicinal properties, including sedative and antispasmodic effects. This study aimed to extract essential oil from lemon leaves and evaluate its antimicrobial potential. The extraction process yielded 0.086% oil, which was subsequently analyzed for its chemical composition and bioactivity. Gas chromatography-mass spectrometry (GC-MS) revealed a diverse array of bioactive compounds, including linalool, citronellal, α-terpineol, citronellol, α-bergamotene, β-cubebene, cis-α-bisabolene, and α-bisabolol, among others. These compounds are associated with antimicrobial, anti-inflammatory, and therapeutic properties. The oil exhibited significant antibacterial and antifungal activity, underscoring its potential as a natural antimicrobial agent. The presence of terpenoids and oxygenated monoterpenes, such as linalool and citronellol, likely contributed to its efficacy against microbial pathogens. These findings highlight the pharmaceutical and nutraceutical value of *C. limon* leaf essential oil, positioning it as a promising candidate for developing natural therapies targeting infections and stress-related disorders. Further research is warranted to optimize extraction methods and validate its mechanisms of action in clinical applications.

KEYWORDS: *Citrus limon*, essential oil, antimicrobial activity, GC-MS analysis, terpenoids, linalool.

INTRODUCTION

The genus *Citrus*, a member of the Rutaceae family, encompasses some of the world's most economically significant fruit-bearing trees and shrubs, including lemon (*Citrus limon*), orange, mandarin, pomelo, grapefruit, and lime. Renowned for their global cultivation, nutritional value, and commercial versatility, citrus fruits account for approximately 37% of total fruit production worldwide, with lemons alone contributing nearly 21% [1-3]. Originating in northeastern India, northern Burma, and southern China, *C. limon* was introduced to the Americas during the late 15th century and is now cultivated extensively in regions such as Chiriqui (Central America) and Pakistan, where citrus production has surged from 563,000 tons in 1973 to 2.3 million tons in 2022, reflecting an annual growth rate of 3.65% [4-5]. The term "lemon" itself traces its linguistic roots through Middle Eastern, Persian, and Sanskrit origins, underscoring its historical and cultural significance [6-8].

Lemons are celebrated not only for their tart flavor but also for their rich phytochemical profile, which includes flavonoids, vitamins (e.g., vitamin C), minerals, dietary fiber, essential oils, and carotenoids [2]. While the fruit is widely studied, the leaves of *C. limon*—small to medium-sized, oval, and glossy with serrated edges—are an underutilized resource. These vibrant green leaves, available year-round, exhibit a unique morphology: a glossy upper surface, matte underside, and a slightly oily texture [9-11]. Beyond their aesthetic appeal, lemon leaves harbor essential oils, concentrated hydrophobic liquids containing volatile aromatic compounds that encapsulate the plant's fragrance and bioactive potential [12].

Essential oils, often comprising 20–300 distinct chemical constituents, are dominated by a few major compounds. For instance, limonene constitutes 60–65% of lemon leaf essential oil (LLEO), while other terpenoids, alcohols, and sesquiterpenes contribute to its antimicrobial, antioxidant, and therapeutic properties [13]. These oils are traditionally extracted via hydro-distillation, a sustainable method utilizing steam to isolate volatile components without chemical solvents. The process, often conducted using a Clevenger apparatus, separates the oil from hydrosol, preserving its integrity for applications in aromatherapy, cosmetics, and natural medicine [14, 15].

Recent studies highlight LLEO's potential as a source of antimicrobial agents, attributed to its high concentrations of linalool, citronellol, and α-terpineol. These compounds disrupt microbial cell membranes and inhibit pathogen growth, positioning LLEO as a promising alternative to synthetic preservatives and antibiotics. Despite these advances, the full scope of its bioactivity, extraction optimization, and practical applications remains underexplored.

This study focuses on the extraction of *C. limon* leaf essential oil via hydro-distillation, its phytochemical characterization using gas chromatography-mass spectrometry (GC-MS), and

evaluation of its antimicrobial efficacy against bacterial and fungal pathogens. By bridging traditional knowledge with modern analytical techniques, this work aims to validate LLEO's role in sustainable agriculture, food preservation, and pharmaceutical innovation.

MATERIAL AND METHOD

REAGENTS AND CHEMICALS

Sodium anhydrous sulphate, distilled water, methanol, hexane, Nutrient agar, potato dextrose agar (PDA), 0.2 % Streptomycin, 1% Fluconazole.

BACTERIA USED

Escherichia coli, Staphylococcus aureus, Salmonella typhi, Bacillus subtilis

FUNGI USED:

Penicillium digitatum, Aspergillus niger, Aspergillus oryzae, Alternaria alternata

COLLECTION OF LEMON LEAVES:

Leaves of citrus limon were collected from PCSIR pilot plant garden. Leaves were washed and cut into small pieces. Filled into round bottom flask with water. The flask is fitted into Clevenger apparatus for hydro distillation.

EXTRACTION OF ESSENTIAL OIL:

Essential oil was extracted from leaves of citrus limon by hydro distillation method. 400 grams of leaves were added into round bottom flask, add 300ml of water, heating the sample for 3 to 4 hours, water and essential oil was collected in glass burette. Water is removed from the stopcock in the beaker and essential oil was collected from the nozzle in vials. Essential oil has some water so add small amount of sodium anhydrous sulphate, that absorb water.

OIL YIELD:

The oil yield refers to the amount of oil extracted from a particular source, such as leaves or plants usually measured in term of weight. The yield can vary depending on factors like the type of source, extraction method. Weight the extracted oil to determine its mass (in grams). Weight the dry leaves to determine its mass (in grams). Calculate the oil yield by using the weight of extracted oil and dry leaves.

GAS CHROMATOGRAPHY AND MASS SPECTROSCOPY ANALYSIS:

SOLVENT USED FOR GCMS:

GC-MS requires volatile organic solvents such as dichloromethane, hexane, methanol, ethyl ether, etc. GC-MS cannot be performed directly on samples dissolved in water.

SAMPLE PREPARATION FOR GCMS:

Sample for GCMS (gas chromatography mass spectroscopy) was prepared in methanol. Fill a GC vial to the 1.5mL mark with a low boiling solvent (e.g. methanol). Add one drop of the sample to be analyzed. If you think you possibly only added a half-drop, it's probably enough.

GCMS TECHNIQUE FOR ESSENTIAL OIL:

The analysis was conducted with an Agilent Technologies 6890 series gas chromatography coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent ChemStation software. A DB-5MS capillary column was used (30m × 0.25mm internal diameter, × 0.25um film thickness). The carrier gas was ultra-pure helium at a flow rate of 1.0 mL/min. The injector temperature was set at 220°C. The initial oven temperature was at 60°C with a hold time of 4

min which was programmed to 260°C at the rate of 10°C/min. Injections of 0.2 µL were made in the split less mode. The mass spectrometer was operated in electron ionization mode at 70eV. Others MS operating parameters were as follows: ion source temperature 230°C quadruple temperature 150°C, solvent delay 4 min and scan range 50-700 amu the compounds were identified by direct comparison 38 of the retention times and mass spectral data and fragmentation pattern with lose in the National Institute of Standards and Technology (NIST) library.

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY:

The agar disc diffusion method was employed for the determination of antibacterial and antifungal activity of essential oil of citrus limon against different food borne pathogens including bacteria (Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Salmonella typhymurium) and fungi (Aspergillus niger, Aspergillus oryzae, Penicillium digitatum, Alternaria alternata). Standard culture media from oxide were employed throughout the present investigation for the purpose of culture maintenance at their respective temperature that is 25°C for fungi and 37°C for bacteria.

ANTIBACTERIAL ANALYSIS:

PREPARE CULTURE MEDIA:

Nutrient agar plates were the most typical growth media for microorganisms. Powder 2.8g of nutrient agar dissolved in 100 ml of distilled water in beaker. Pour the media into flask. Boiled it to dissolve completely in a water bath. Autoclave the media when ingredients fully dissolve. Sterilized by autoclaving at 121°C for 15 minutes. Cools down the media and pours into sterile petri dishes for solidification.

Figure 2. Culture media

Sterilized petri dishes labelled with given bacteria. Add nutrient agar and bacteria one by one in a labelled Petri dish and leave for 20 to 30 min to solidify and for bacteria to grow.

Figure 3. Bacterial culture media plates

Sterile and dried 4mm paper discs were impregnated with filtered and sterilized extracted essential oil and placed on freshly seeded bacterial culture media with a control. The petri plates

were incubated at 37°C for 24 hours and zones of inhibition thus developed against tested microorganisms were measured in millimeters. 0.2% streptomycin used as standard point.

ANTIFUNGAL ANALYSIS:

PREPARE CULTURE MEDIA:

Potato dextrose agar (abbreviated "PDA") is the most widely used medium for growing fungi and bacteria. Dissolve powder 3.9g of potato dextrose agar (PDA) in 100 ml of distilled water. Pour the media into flask. Boiled it to dissolve completely in a water bath. Autoclave the media when ingredients are fully dissolved. Sterilized by autoclaving at 121°C for 15 minutes. Cools down the media and pours into sterile petri dishes for solidification.

Sterilized the petri dishes and labelled with given fungi. Add potato dextrose agar and fungi one by one in a labelled Petri dish and leave for 20 to 30 min to solidify and for bacteria to grow.

Figure 4. Fungal culture media plates

Sterile and dried 4mm paper discs were impregnated with filtered and sterilized extracted essential oil and placed on freshly seeded fungal culture media with a control. The petri plates were incubated at 37°C for 24 hours and zones of inhibition thus developed against tested microorganisms were measured in millimeters. 1% Fluconazole used as standard point.

Zone of Inhibition test (also referred to as Kirby-Bauer test) was used to determine the susceptibility or resistance of pathogenic bacteria to antibacterial agents. This was an area of media where bacteria were unable to grow, due to the presence of an essential oil that impedes their growth. It was a clear circular area around antimicrobial discs in which bacteria were unable to grow.

RESULTS

YIELD OF ESSENTIAL OIL:

Essential oil of lemon leaves was extracted by hydro distillation. The obtained oil was measured in grams, and the percentage was calculated. The yield of the hydro distillation essential oil of lemon leaf was 0.086% and it was characterized by a pale yellowish color and pleasant aromatic fragrance.

Total leaves = 3500g

Mass of extracted oil = 3g

% Yield = Mass of extracted oil ÷ Mass of total leaves

= 3g ÷ 3500g = 0.086 %

CHEMICAL COMPOSITION:

The essential oil was extracted by hydro distillation using Clevenger apparatus. The percentage yield was 0.086% of *limon*. The chemical analysis revealed that essential oil is mainly constituted of monoterpenes while the alcohols, aldehydes and esters were found in small amounts. Based on our findings, the main constituents of lemon leaf essential oil are linalool (10.03%), R-citronellol (25.48%), β-Bisabolene (13.54%) and α-terpineol (7.99%).

Table 4.1. Components present in EO of lemon leaves

Sr. #	Retention time	Compound Found	Area
1	3.998	1,6-Octadiene-3-ol, 3,7-dimethyl-	10.03
2	4.592	Isopulegol	2.16
3	4.674	Citronellal	5.39
4	5.164	α-Terpineol	7.99
5	5.604	Citronellol	1.93
6	5.676	6-Octen-1-ol,3,7-dimethyl-, (R)-	25.48
7	8.117	Bicyclo[3.1.1] hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentyl)-	5.80
8	8.686	1H-Cyclopenta[1,2]cyclopropa[1,2] benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,3aS- (	2.32
9	8.976	Cis-α-Bisabolene	2.05
10	9.039	β-Bisabolene	13.54
11	10.309	Cubenol	1.96
12	10,794	α-Cadinol	5.92
13	10.942	1,4-Methanoazulene-9-methanol, decahydro-4,8,8-trimethyl-, [1S-(1α,3aβ,4α,	7.18
14	11.130	α-Bisabolol	8.18

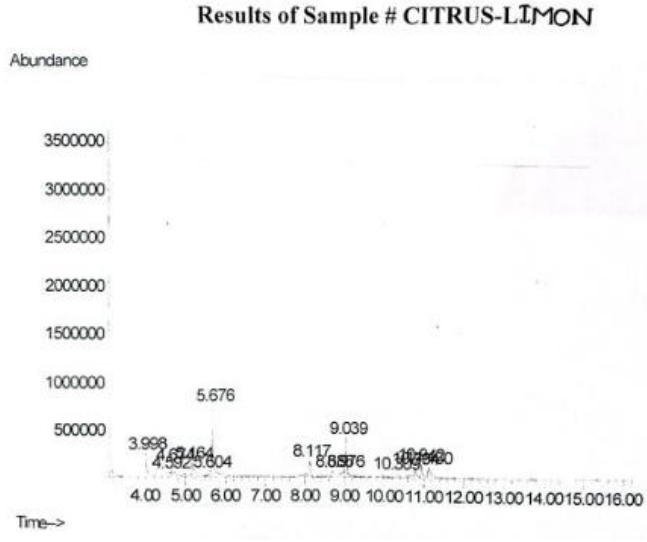

Figure 4.4. Analysis of lemon leaves essential oil

ANTIBACTERIAL ACTIVITY:

The essential oil of citrus limon leaves showed good activity against all bacterial strains i.e. Gram positive (S. aureus, B. subtilis) and Gram negative (E. coli, S. typhi). 0.2 % streptomycin used as standard.

Table 4.2. Zone of inhibition of different bacteria

Bacteria	Standard	Inhibition zone	
		24 hours	48hours
Escherichia coli	22mm	20mm	20mm
Staphylococcus aureus	21mm	25mm	21mm
Salmonella typhymurium	24mm	20mm	21mm
Bacillus subtilis,	13mm	08mm	19mm

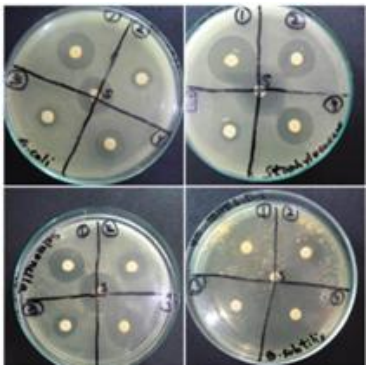

Figure 4.5. Inhibition zone of a) E. coli, b) S. aereus c) S. typhi d) B. subtilis

S = Standard (0.2 % Streptomycin), 1 = I.Z of essential oil of citrus limon

ANTIFUNGAL ACTIVITY:

The essential oil of citrus limon leaves showed good activity against all fungal strains i.e. A. niger, A. oryzae, P. digitatum, A. alternata. They show inhibition zones of 20mm, 22mm, 24mm, 20mm respectively. Highest zone of inhibition showed by P. digitatum. 1% fluconazole used as standard.

Table 4.3. Zone of inhibition of different fungi

Fungi	Standard	Inhibition Zone	
	1%Fluconazole	24 hours	48 hours
Aspergillus niger	11mm	20mm	18mm
Aspergillus oryzae	16mm	22mm	21mm
Penicillium digitatum	18mm	24mm	22mm
Alternaria alternata	16mm	20mm	19mm

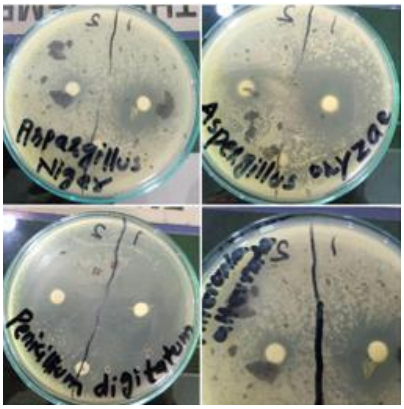

Figure 4.6. Inhibition zone of a) A. niger, b) A. oryzae, c) P. digitatum, d) A. alternata

S = Standard 1 = I.Z of essential oil of citrus limon

Table 4.4. Comparison of antimicrobial activity of LLEO against different microbes

Tested Microorganism	Incubation temp. (°C)	Culture media	Colony morphology	Inhibition zone (mm)
E.coli	37°C	CM021	White, later grayish	20mm
S.aureus	37°C	CM021	Yellowish	25mm
S. typhymurium	37°C	CM021	Off-white	20mm
B.subtilis	37°C	CM021	Yellowish	08mm
A.niger	25°C	CM139	White, later green/black	20mm
A.oryzae	25°C	CM139	White, later green/black	22mm
P.digitatum	25°C	CM139	White, later green	24mm
A.alternata	25°C	CM139	Grayish	20mm

CM021: Nutrient agar 20mm, CM139: Potato dextrose agar

DISCUSSION

Citrus limon (lemon) leaf essential oil (LLEO) exhibits notable antimicrobial, antibacterial, and antifungal properties, alongside therapeutic benefits for skin health and emotional well-being. Traditionally used to alleviate anxiety, uplift mood, and treat skin conditions like acne, LLEO demonstrates efficacy when diluted and applied topically. Its antibacterial action targets pore-trapped bacteria, reducing breakouts, while its exfoliating properties help clarify skin by removing dead cells from hair follicles and pores.

In this study, LLEO was extracted via hydro-distillation using a Clevenger apparatus, yielding 0.086% oil. GC-MS analysis revealed a complex phytochemical profile dominated by monoterpenes, sesquiterpenes, and oxygenated derivatives, including **linalool (10.03%)**, **R-citronellol (25.48%)**, **β-bisabolene (13.54%)**, **α-bisabolol (8.18%)**, **α-terpineol (7.99%)**, and **longifolol (7.18%)**. These compounds, particularly linalool and R-citronellol, are strongly associated with antimicrobial activity.

LLEO demonstrated **robust antimicrobial effects**, classified as "very strong" against bacterial strains such as *Escherichia coli* (20 mm inhibition zone), *Staphylococcus aureus* (25 mm), *Salmonella Typhimurium* (20 mm), and *Bacillus subtilis* (8 mm). Antifungal activity was equally significant, with inhibition zones of 20–24 mm against *Aspergillus niger*, *A. oryzae*, *Penicillium digitatum*, and *Alternaria alternata*. Notably, *S. aureus* (bacteria) and *P. digitatum* (fungus) exhibited the largest inhibition zones, underscoring LLEO's broad-spectrum potential.

The findings position LLEO as a promising natural alternative to synthetic preservatives and antibiotics. Its ability to inhibit microbial growth highlights applications in food preservation and pharmaceutical formulations. Future research should focus on optimizing extraction yields, elucidating molecular mechanisms of action, and validating safety in *in vivo* models. Harnessing LLEO's bioactive properties could address rising antibiotic resistance and consumer demand for eco-friendly solutions.

CONCLUSIONS

The present study successfully demonstrated the extraction of essential oil from *Citrus limon* leaves using an eco-friendly hydro-distillation method, yielding 0.086% oil. The application of GC-MS analysis revealed a chemically diverse profile dominated by linalool, a key bioactive compound, alongside other terpenoids and oxygenated monoterpenes such as citronellal, α-terpineol, and α-bisabolol. The essential oil exhibited significant antimicrobial activity against bacterial and fungal pathogens, attributed to its unique phytochemical composition. The high concentration of linalool, known for its antimicrobial and anti-inflammatory properties, likely plays a pivotal role in the oil's bioactivity. These findings underscore the potential of *C. limon* leaf essential oil as a sustainable, natural alternative to synthetic preservatives and antibiotics. Its efficacy in inhibiting microbial growth highlights its applicability in the food industry as a preservative and in pharmaceuticals for developing novel therapeutic agents. Future studies should focus on elucidating the precise mode of action of the oil against microbial cells, optimizing extraction protocols for higher yields, and evaluating its safety and efficacy in *in vivo* models. Harnessing the untapped potential of *C. limon* leaves could pave the way for innovative solutions to combat antibiotic resistance and meet the growing demand for natural, eco-friendly products.

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