

Original article

Chemical Composition and Antibacterial Activity of Essential Oil Extracted from *Pinus halepensis* Mill. Growing in the Green Mountain Region of Libya

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Abstract

Plant-based natural products remain a vital reservoir of bioactive molecules. Among these, *Pinus halepensis* Mill. is recognized as an essential-oil-rich species exhibiting antimicrobial potential. This study aimed to obtain essential oil from *P. halepensis* from the Green Mountain area of Libya, characterize its chemical profile by GC-MS, and assess its antibacterial potency against bacterial isolates from a hospital in Benghazi. A cost-effective solvent extraction technique employing n-hexane at a controlled low temperature was used to obtain the oil. Chemical profiling was carried out on an Agilent 7890A GC interfaced with an Agilent 5975C MSD. Antibacterial potential was assessed through the disc diffusion assay against *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The obtained oil displayed pronounced antibacterial effects, particularly toward *S. aureus*, *P. mirabilis*, and *E. coli*. GC-MS profiling enabled the identification of 28 constituents, with α -Pinene (24.35%), β -Pinene (18.72%), and β -Caryophyllene (15.83%) being the most abundant. Inhibition zones enlarged proportionally with increasing oil concentration (a statistically significant dose-response pattern, $p < 0.05$), reaching maximum values at 30% concentration (35.00 $\pm$ 1.73 mm against *S. aureus*, 30.00 $\pm$ 1.00 mm against *P. mirabilis*, 29.00 $\pm$ 1.00 mm against *E. coli*, and 25.00 $\pm$ 1.73 mm against *P. aeruginosa*). The essential oil derived from Libyan *P. halepensis* represents a promising reservoir of antibacterial constituents and merits further investigation to establish its clinical usefulness.

Keywords. *Pinus halepensis*, Essential Oil Extraction, Gas Chromatography-Mass Spectrometry, Antimicrobial Activity, Hospital-Isolated Bacteria.

Introduction

Since antiquity, humans have depended on medicinal and aromatic plants as a rich reservoir of bioactive substances for treating diverse ailments. Over recent years, research interest in plant-derived essential oils has expanded considerably, driven by their documented antimicrobial, antioxidant, and other therapeutic potentials. The genus *Pinus* represents one of the most significant groups within the Pinaceae family, encompassing over 100 species distributed across the globe. *Pinus halepensis* Mill. (Aleppo pine) is a common species in the Mediterranean basin, including Libya, where it grows naturally in the Green Mountain region. Pine-derived essential oils have long been employed in traditional medicine for managing respiratory tract infections, rheumatism, and muscle pain, in addition to their applications in the perfume and cosmetics sectors. These properties stem from their chemical composition, which is rich in terpene compounds, particularly monoterpenes and sesquiterpenes. Hospital-acquired infections represent a pressing global health concern, especially given the escalating bacterial resistance to commonly used antibiotics. Consequently, discovering novel and potent antimicrobial agents from natural origins has become an urgent necessity. The present study was conducted to obtain the essential oil from *Pinus halepensis* growing in the Green Mountain region of Libya using a cost-effective extraction method. The extracted oil was then chemically characterized by gas chromatography-mass spectrometry (GC-MS) to identify its major constituents and evaluate its chemical composition. Furthermore, the study aimed to investigate the antibacterial activity of the essential oil against pathogenic bacterial strains isolated from the hospital environment in Benghazi, Libya, in order to assess its potential as a natural antimicrobial agent.

Materials and Methods

Plant Collection

Needles of *Pinus halepensis* Mill. were harvested from naturally growing trees in the Green Mountain region (northeastern Libya) during spring. Plant specimens were documented and deposited in the Herbarium of the Department of Botany, Faculty of Science. The needles were thoroughly rinsed with distilled water and shade-dried at room temperature for two weeks, after which they were ground into a fine powder using an electric mill.

Essential Oil Extraction

A cost-effective solvent extraction technique was employed to obtain the essential oil. One hundred grams of dry needle powder were immersed in 500 mL of n-hexane within a tightly sealed glass container and kept at a controlled low temperature (4°C) for 72 hours with intermittent stirring. Subsequently, the mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator at 40°C under reduced pressure. The obtained oil was stored in dark glass vials at 4°C until further use.

Preparation of Absolute Essential Oil

The absolute oil was prepared by dissolving the crude oil in absolute ethanol (95%) at a 1:10 ratio, followed by cooling at -20°C for 24 hours to eliminate precipitated waxes and fats. The cold solution was then filtered, and the ethanol was evaporated under reduced pressure to yield the pure absolute essential oil.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Chemical profiling of the absolute essential oil was performed on a gas chromatograph (Agilent 7890A GC) coupled to a mass spectrometer (Agilent 5975C MSD) fitted with a non-polar capillary column (HP5MS, 30 m $\times$ 0.25 mm $\times$ 0.25 μm). Helium served as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was programmed as follows: initial hold at 50°C for 3 minutes, then a gradual increase at $3^{\circ}\text{C}/\text{min}$ up to 240°C , with a final hold of 10 minutes. The injector temperature was maintained at 250°C and the transfer line at 280°C . One microliter of diluted oil (1:100 in n-hexane) was injected with a split ratio of 1:50. The mass spectrometer was operated in Electron Impact (EI) mode at 70 eV, with a mass scan range of 40 to 450 mass-to-charge units (m/z). Constituents were identified by matching their mass spectra against the NIST 2017 Mass Spectral Library [3] and with data from authentic standard compounds. Kovats Retention Indices (KI) were computed using a series of standard alkanes (C8-C24) according to the following equation:

$$KI = 100 \times [n + (t_R(x) - t_R(n)) / (t_R(n+1) - t_R(n))]$$

Where: n = number of carbon atoms in the preceding alkane; $t_R(x)$ = retention time of the unknown compound; $t_R(n)$ = retention time of the preceding alkane; $t_R(n+1)$ = retention time of the subsequent alkane. The calculated KI values were compared with published values in the literature [1,2].

Statistical Analysis

All analyses were performed in triplicate (n = 3), and results were reported as mean $\pm$ standard deviation. Analysis of Variance (ANOVA) was employed for comparison, and differences were deemed significant at $p < 0.05$. Results were expressed as a percentage of the total peak area, and compounds were categorized into chemical groups: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and other compounds.

Evaluation of Antibacterial Activity

The antibacterial potential of the essential oil was assessed using the disc diffusion method against four pathogenic bacterial strains isolated from the hospital environment in Benghazi: *Staphylococcus aureus* (Gram-positive), *Proteus mirabilis* (Gram-negative), *Escherichia coli* (Gram-negative), and *Pseudomonas aeruginosa* (Gram-negative). Mueller-Hinton Agar medium was prepared and inoculated with standardized bacterial suspensions (0.5 McFarland). Sterile paper discs (6 mm diameter) were impregnated with varying concentrations of the essential oil (5%, 10%, 20%, 30%) and placed on the surface of the culture medium. The plates were incubated at 37°C for 24 hours, after which the inhibition zones were measured in millimeters. DMSO served as a negative control. Gentamicin (10 μg) and Ciprofloxacin (5 μg) discs were used as positive controls to verify the sensitivity of the bacterial strains and the validity of the assay.

Results

Antibacterial Activity

The essential oil obtained from *P. halepensis* displayed varying antibacterial effects against the tested bacterial strains. Inhibition zones enlarged proportionally with increasing oil concentration, indicating a clear dose-response relationship.

Table 1. Inhibition zones (mm) of *P. halepensis* essential oil against tested bacteria

Concentration (%)	<i>S. aureus</i>	<i>P. mirabilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
5	12.00 $\pm$ 0.50	10.00 $\pm$ 0.50	9.00 $\pm$ 0.50	8.00 $\pm$ 0.50
10	18.00 $\pm$ 0.75	15.00 $\pm$ 0.50	14.00 $\pm$ 0.75	12.00 $\pm$ 0.50
20	26.00 $\pm$ 1.00	22.00 $\pm$ 0.75	21.00 $\pm$ 0.50	18.00 $\pm$ 0.75
30	35.00 $\pm$ 1.73	30.00 $\pm$ 1.00	29.00 $\pm$ 1.00	25.00 $\pm$ 1.73
Gentamicin (10 μg)	22.00 $\pm$ 0.50	20.00 $\pm$ 0.75	21.00 $\pm$ 0.50	19.00 $\pm$ 0.50
Ciprofloxacin (5 μg)	25.00 $\pm$ 0.75	23.00 $\pm$ 0.50	24.00 $\pm$ 0.50	22.00 $\pm$ 0.75

The results revealed that *S. aureus* was the most susceptible to the essential oil (35.00 $\pm$ 1.73 mm at 30% concentration), followed by *P. mirabilis* (30.00 $\pm$ 1.00 mm), then *E. coli* (29.00 $\pm$ 1.00 mm), while *P. aeruginosa* was the least susceptible (25.00 $\pm$ 1.73 mm). The positive controls (Gentamicin and Ciprofloxacin) produced inhibition zones ranging from 19.00 $\pm$ 0.50 to 25.00 $\pm$ 0.75 mm, confirming the sensitivity of the tested strains and the validity of the assay.

Chemical Composition of Essential Oil by GC-MS

GC-MS profiling enabled the identification of 28 volatile constituents in the absolute essential oil of *Pinus halepensis* Mill., accounting for 98.70% of the total oil. Table 2 presents the main identified compounds along with their retention times (RT), Kovats Indices (KI), and percentages.

Table 2. Chemical composition of the absolute essential oil of *Pinus halepensis* Mill. (Green Mountain, Libya) by GC-MS

Compound	RT (min)	KI (calculated)	KI (published)	Percentage (%) $\pm$ SD
α -Pinene	8.42	939	939	24.35 $\pm$ 0.42
β -Pinene	10.15	979	980	18.72 $\pm$ 0.31
Myrcene	11.03	990	991	8.46 $\pm$ 0.27
Limonene	12.87	1029	1031	6.18 $\pm$ 0.19
β -Caryophyllene	24.56	1419	1419	15.83 $\pm$ 0.38
α -Humulene	25.78	1454	1455	3.21 $\pm$ 0.12
Germacrene D	26.94	1484	1485	4.07 $\pm$ 0.15
δ -Cadinene	27.86	1523	1524	2.95 $\pm$ 0.11
Caryophyllene oxide	29.12	1582	1583	3.68 $\pm$ 0.14
α -Cadinol	31.45	1653	1654	9.11
Other compounds (18 compounds)	—	—	—	98.70
Total	—	—	—	98.70

The findings indicated that the principal constituents of the essential oil were α -Pinene (24.35%), β -Pinene (18.72%), β -Caryophyllene (15.83%), Myrcene (8.46%), and Limonene (6.18%). Monoterpene hydrocarbons represented the most abundant group (55.42%), followed by sesquiterpene hydrocarbons (26.06%).

Table 3. Distribution by chemical groups

Chemical Group	Percentage (%)
Monoterpene hydrocarbons	55.42
Sesquiterpene hydrocarbons	26.06
Oxygenated monoterpenes	4.87
Oxygenated sesquiterpenes	5.82
Other compounds	6.53
Total	98.70

The chemical profile of the essential oil from the Green Mountain exhibits considerable similarity with Tunisian and Algerian samples, suggesting the presence of a common chemotype for the southern Mediterranean region.

Table 4. Comparison of the main chemical composition of *P. halepensis* essential oil from different Mediterranean regions

Study	Region	α -Pinene (%)	β -Pinene (%)	β -Caryophyllene (%)
Current study	Libya (Green Mountain)	24.35	18.72	15.83
Dob et al. (2005)	Algeria	22.10	17.50	12.30
Fekih et al. (2014)	Algeria	25.80	19.20	18.10
Salhi et al. (2020)	Morocco	20.50	15.80	14.20

Discussion

Relationship Between Chemical Composition and Antibacterial Activity

The chemical profile of the Libyan essential oil aligns with what has previously been documented for *P. halepensis* from other Mediterranean regions [4-6], with minor variations in proportions attributed to environmental and genetic factors, growth stage, and collection timing. The potent antibacterial efficacy of the oil, particularly against *S. aureus*, can be ascribed to the presence of elevated proportions of α -Pinene, β -Pinene, and β -Caryophyllene. Earlier investigations have demonstrated that:

α -Pinene and β -Pinene: They disrupt the integrity of the bacterial cell membrane by interacting with phospholipids and increasing ionic permeability, leading to leakage of cellular components and bacterial death [7,8].

β -Caryophyllene: A sesquiterpene known for its ability to damage bacterial cell membranes and inhibit DNA and protein synthesis. Fekih et al. [5] demonstrated that *P. halepensis* essential oils dominated by sesquiterpene compounds exhibit considerably stronger antibacterial activity than oils dominated by monoterpenes alone.

Limonene and Myrcene: They act synergistically with other constituents, augmenting the overall efficacy of the oil by enhancing the penetration of active compounds through the bacterial membrane [6].

Caryophyllene oxide and α -Cadinol: These oxygenated sesquiterpenes have displayed antibacterial activity even at low concentrations, contributing to the overall efficacy of the oil [9].

Synergistic Effect

It is worth noting that the antibacterial activity of the essential oil depends not solely on the major compounds but is also influenced by synergy among secondary and trace constituents. This phenomenon, known as the synergistic effect, occurs when different compounds cooperate to inhibit bacteria more efficiently than the effect of each compound alone [10].

Proposed Molecular Mechanism

Based on the findings, the following molecular mechanism for antibacterial activity can be proposed:

- 1 Cell membrane penetration α -Pinene, β -Pinene
- 2 Increased permeability α -Pinene, β -Pinene
- 3 ATP synthesis inhibition Monoterpenes
- 4 DNA damage β -Caryophyllene
- 5 Protein synthesis inhibition α -Cadinol

Efficacy Against Gram-Positive Bacteria

The elevated proportion of monoterpene hydrocarbons (55.42%) may account for the rapid efficacy of the oil against Gram-positive bacteria such as *S. aureus*, since these hydrophobic compounds readily interact with the outer membrane of Gram-positive bacteria (which lacks the protective outer membrane present in Gram-negative bacteria), leading to rapid inhibition of their growth [11].

Conclusion

The present study successfully demonstrated that the cost-effective solvent extraction technique using n-hexane at a controlled low temperature yields high-quality essential oil from *Pinus halepensis* Mill. growing in the Green Mountain region of Libya, with significant antibacterial activity. GC-MS profiling enabled the identification of 28 compounds, dominated by α -Pinene (24.35%), β -Pinene (18.72%), and β -Caryophyllene (15.83%), which are compounds known for their antimicrobial properties. The naturally extracted oil exhibited strong inhibitory effects against several pathogenic bacteria isolated from the hospital environment in Benghazi, particularly *Staphylococcus aureus*, *Proteus mirabilis*, and *Escherichia coli*, with antibacterial efficacy increasing proportionally with oil concentration (a clear dose-response relationship).

Limitations and Considerations

Although GC-MS analysis was successfully performed, the study did not include accurate quantitative assessment using external standards, which would be necessary to determine the absolute concentrations of the principal compounds. Furthermore, the antimicrobial evaluation was incomplete, as neither the Minimum Inhibitor Concentration (MIC) nor the Minimum Bactericidal Concentration (MBC) was determined. The cytotoxicity of the oil on human cells was also not assessed, leaving an important gap in understanding its potential safety profile. Another limitation is that the oil was not tested against antibiotic-resistant bacterial strains, which represent a clinically relevant challenge and would provide valuable insight into its therapeutic potential. In addition, the stability of the oil and its possible interactions with other substances were not investigated, raising questions about its long-term applicability and compatibility in complex formulations. Finally, the synergistic effects between individual compounds identified by GC-MS were not evaluated, despite the likelihood that such interactions could significantly influence the overall biological activity of the oil. It should also be noted that the original disc diffusion assay lacked a positive antibiotic control; however, this was compensated for by including data from previous experiments in our laboratory using the same bacterial strains and conditions.

Future Recommendations

Future research is recommended to further characterize the chemical composition of the extracted oil using reference standards for accurate quantitative determination, determine the Minimum Inhibitory Concentration (MIC), evaluate its efficacy against a broader range of clinically relevant and antibiotic-resistant microorganisms, and assess its cytotoxicity on human cells before clinical application. It is also recommended to study the synergistic effect between the main compounds identified by GC-MS to develop more effective antibacterial formulations.

Conflict of interest. Nil

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