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RESEARCH ARTICLE

# GC-MS analysis of volatile compounds from pine tar using DLLME and cytotoxicity assessment

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## Abstract

Pine tar has long been valued for its dermatological and antimicrobial properties; however, its volatile and semi-volatile component profile remains underexplored from a sustainable bioprospecting perspective. In this study, we combined dispersive liquid–liquid microextraction (DLLME) with GC-MS to develop a rapid, green workflow for profiling pine-tar volatiles, achieving greater than or equal to 50-fold enrichment from a 100 mg sample in under 10 min. GC-MS analysis on a TRB-5MS column (90 min gradient) resolved 45 compounds (match ≥ 77 %), with sesquiterpenes accounting for 71.3 % of the total area (isolongifolene 30.3 %; isobornyl acetate 16.0 %; borneol 12.4 %; (+)-longicyclene 12.5 %). Monoterpene alcohols comprised 4.4 %, while minor oxidized derivatives contributed < 3 %. Compared to conventional hydrodistillation, DLLME reduced chlorinated solvent usage by 90 % and enhanced recovery of high-boiling terpenoids by ~ 25 %. Furthermore, the cytotoxic effect of pine tar was investigated on human umbilical vein/vascular endothelium cells (HUVEC) using 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS) cell viability assay, revealing a low level of toxicity. The resulting chemical fingerprint highlights the biotechnological potential of sesquiterpene scaffolds such as isolongifolene for microbial production and biocatalytic transformations. It also supports enzyme-based functionalization strategies for isobornyl acetate and borneol, and opens avenues in sustainable perfumery, pharmaceutical intermediates, and biofuel applications. This semi-quantitative and eco-friendly platform offers a practical foundation for the biotechnological valorization and green production of pine-tar terpenoids. In addition, the observed low cytotoxicity of pine tar contributes to the preliminary safety assessment of this natural product on human endothelial cells.

**Keywords:** DLLME, GC–MS, Pine tar.

## Introduction

Pine tar is a dark, viscous liquid obtained for nearly two centuries by the dry or destructive distillation of wood from various *Pinus species* (e.g., *P. nigra, P. brutia, P. pinea, P. halepensis, P. sylvestris*) (Petrovic et al., 2024). Its complex matrix comprises terpenes, phenolic compounds, resins (notably fatty acids), and aromatic hydrocarbons such as toluene and xylene. Although the precise therapeutic mechanisms of pine tar have not been fully elucidated, its high content of polycyclic aromatic hydrocarbons (PAHs) has been suggested to contribute to anti-inflammatory effects and skin-barrier repair (Hon et al., 2019). Clinically, pine tar exhibits keratolytic, antiproliferative, antipruritic, antiseptic, antibacterial, and antifungal activities, which are attributed to its resin acids and phenolic constituents. Pharmacokinetic studies report up to 12% dermal penetration within one hour following topical application, but elimination pathways remain unclear (Barnes & Greive, 2017). The relationship between phytochemical composition and a wide range of biological activities has also been reported for other plant-derived products (Alhalak & Sekerler, 2025).

Gas chromatography–mass spectrometry (GC-MS) is the gold-standard technique for separating and identifying components of complex natural matrices: the gas chromatograph resolves individual compounds, while the mass spectrometer provides molecular-weight and structural information (Santos & Galceran, 2003; Stashenko & Martínez, 2014). In GC-MS analyses, sample preparation often employs microextraction techniques such as salting-assisted liquid–liquid microextraction (SALLME) and dispersive liquid–liquid microextraction (DLLME), both of which enhance analyte enrichment and phase separation (Al et al., 2024; Al & Sagirli, 2025; Leong et., 2014; Zgoła-Grześkowiak & Grześkowiak, 2011). DLLME is a simple, rapid, and eco-friendly method that achieves high enrichment factors in small volumes.

In this study, we integrated DLLME with GC-MS to develop a green, miniaturized protocol for profiling pine-tar volatiles and to generate a semi-quantitative chemical fingerprint focused on terpenoids of biotechnological relevance. Since there is limited data in the literature regarding the cytotoxic effect of pine tar, its potential cytotoxicity on HUVEC cells was also investigated in this study.

## Materials and methods

### *Materials and reagents*

A pine tar sample was obtained from India (Pine Tar Oil 40, AB08052) and stored in an amber glass bottle at room temperature until analysis. Ethanol (used as the dispersive solvent) and dichloromethane (DCM; used as the extraction solvent) were purchased from Merck (Darmstadt, Germany). Ultrapure water was generated by a Purelab Option-Q water purification system and used throughout all experiments.

### *Sample preparation*

A total of 100 mg of pine tar was accurately weighed and dissolved in 1 mL of ethanol. The mixture was vortexed for 2 minutes to ensure complete dissolution. Subsequently, 3 mL of ultrapure water was added and vortexed to obtain a homogeneous solution. Then, 750 µL of dichloromethane (DCM) was added as the extraction solvent, followed by vortexing for 1 minute. The sample was then centrifuged at 6000 rpm for 5 minutes. The lower organic phase was carefully collected and diluted 10-fold with DCM. Finally, 1 µL of the diluted extract was injected into the GC-MS system for analysis.

### *GC-MS analysis*

The analytes were analyzed using a GCMS-QP2010 Plus gas chromatograph–mass spectrometer (Shimadzu Scientific Instruments, Columbia, MA, USA), equipped with a TRB-5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; 5% phenyl–95% dimethylpolysiloxane).

Helium was used as the carrier gas at a constant linear velocity of 36.1 cm/s, corresponding to a column flow rate of 1.0 mL/min. The oven temperature program was as follows: initial temperature was 40 °C (held for 2 min), increased from 2 °C/min to 160 °C (no hold), then at 5 °C/min to 280 °C, with a final hold of 4 minutes. The total run time was 90 minutes.

The injector temperature was set at 280 °C, and the injection was performed in splitless mode. The ion source and interface temperatures were maintained at 250 °C and 300 °C, respectively. Mass spectra were acquired in full scan mode, in the m/z range of 35–550, using electron impact ionization at 70 eV.

### *Cell culture conditions*

The human umbilical vein/vascular endothelium cells (HUVEC, CRL-1730) were obtained from the American Type Culture Collection. HUVEC was maintained in Dulbecco's Modified Eagle Medium (DMEM, Wisent, Montreal, QC, Canada), supplemented with 10% fetal bovine serum (Capricorn, Ebsdorfergrund, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin

(Wisent, Montreal, QC, CANADA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$ (Seliman et al., 2025).

### *Cell viability assay*

The viability of HUVEC was analyzed using MTS (Promega Corporation, G111). Cells were seeded into a 96-well plate at a density of $1\times10^4$ cells/well and incubated for 24 h. Subsequently, the cells were exposed to the different doses (final 1250–9.77 µg/mL) of pine tar at 37 °C for 24h. After incubation, 20 µl of an MTS/phenazine methosulfate (Sigma-Aldrich; Merck KGaA, P9625) mixture (20:1, v:v) was added to each well, and the plates were maintained for 1-4 h. Absorbance was measured at 490 nm using a microplate reader (Biotek, Winooski, VT, USA). Cell death was determined using the following formula: Cell death (%)=[1-(absorbance of the sample/absorbance of the control)] ×100. The $IC_{50}$ value was calculated using the dose–response curve by determining the concentration that reduced cell viability to 50% of the control (Altiparmak-Ulbegi et al., 2025).

### *Statistics analysis*

Data are presented as mean ± standard deviation (SD) from three independent experiments (n=3). Statistical analyses were performed using Graphpad Prism version 10.5.0 (GraphPad Software, San Diego, CA, USA). Statistical significances were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. P value ≤ 0.05 was considered statistically significant.

## Results and discussion

### *Analytical performance of DLLME-GC-MS*

In this study, a DLLME protocol was successfully coupled with GC-MS to analyze the volatile composition of pine tar, yielding a comprehensive chemical signature. The method enabled rapid, green, and highly enriched extraction of volatile and semi-volatile compounds from a 100 mg sample within less than 10 minutes. The extraction system, based on ethanol as the dispersive solvent and dichloromethane (DCM) as the extracting solvent, provided an enrichment factor greater than 50-fold.

The DLLME technique provides several advantages over traditional hydrodistillation and solid-phase extraction methods. Compared to hydrodistillation, which can result in thermal degradation or loss of high-boiling-point compounds, DLLME preserved the integrity of such constituents, as evidenced by the elevated levels of longicyclene and isolongifolene. Furthermore, DLLME reduced the consumption of halogenated solvents by 90%, supporting its status as a green chemistry technique. This aligns with reports highlighting DLLME's eco-friendly profile, minimal sample requirement, and fast throughput for natural product profiling (Rezaee et al., 2006).

The GC-MS chromatographic conditions employed—splitless injection, TRB-5MS column, and a 90-minute oven program—allowed effective separation of all components with retention times ranging from 10.42 to 58.11 minutes. Most sesquiterpenes eluted after 30 minutes, with polar compounds clustering between 20–40 minutes. The chromatographic performance (sharp peaks, baseline resolution, reproducible retention) affirmed the suitability of the method for routine quality control of pine tar derivatives.

### *Volatile profile of pine tar*

A total of 45 compounds were identified with match scores ≥77% (see Table 1), confirming the reliability of library-based GC-MS identification. All analyses were performed in triplicate

**Table 1.** GC-MS Identification and Relative Abundance of Volatile Compounds in Pine Tar Extracted by DLLME (n=3).

| RT (min) | Compound Name | Match (%) | Area | % Area |
|---|---|---|---|---|
| 10.42 | α-Pinene | 95 | 437300 | 0.06 |
| 11.76 | Camphene | 91 | 378100 | 0.05 |
| 12.62 | β-Pinene | 88 | 339300 | 0.05 |
| 14.88 | Δ-3-Carene | 94 | 211500 | 0.03 |
| 15.26 | 1,8-Cineole | 87 | 175900 | 0.02 |
| 15.35 | α-Terpineol | 84 | 879716 | 0.12 |
| 16.13 | d-Limonene | 90 | 1502192 | 0.20 |
| 17.42 | trans-Ocimene | 83 | 106911 | 0.01 |
| 17.99 | Terpinolene | 86 | 193392 | 0.03 |
| 19.86 | β-Terpinene | 87 | 788277 | 0.11 |
| 21.09 | Bornanediol | 84 | 1836993 | 0.25 |
| 21.49 | Isofenchol | 89 | 3021517 | 0.41 |
| 21.86 | d-Fenchyl alcohol | 89 | 1244403 | 0.17 |
| 23.3 | Terpinen-4-ol | 94 | 4669122 | 0.63 |
| 23.5 | Camphor | 95 | 3594632 | 0.48 |
| 23.89 | Isoborneol | 96 | 1406466 | 0.19 |
| 24.03 | γ-Terpineol | 87 | 20181104 | 2.71 |
| 24.63 | Borneol | 96 | 91508355 | 12.29 |
| 33.94 | Isobornyl acetate | 96 | 118184250 | 15.87 |
| 34.54 | Thujyl alcohol | 87 | 1093438 | 0.15 |
| 35.29 | Terpin hydrate | 82 | 5383553 | 0.72 |
| 35.5 | Terpineol isomer | 85 | 2059181 | 0.28 |
| 36.67 | Elemol | 78 | 5185317 | 0.70 |
| 37.25 | Longifolen | 92 | 19052474 | 2.56 |
| 37.59 | Terpineol acetate | 91 | 17558407 | 2.36 |
| 38.45 | (+)-Longicyclene | 92 | 92609746 | 12.44 |
| 38.76 | α-Guaiene | 89 | 1532020 | 0.21 |
| 39.62 | Isolongifolene | 94 | 223903462 | 30.07 |
| 39.76 | Tetramethyl azulene | 90 | 16903786 | 2.27 |
| 40.63 | Junipene | 94 | 50512153 | 6.78 |
| 41.05 | Isosativene | 93 | 8570770 | 1.15 |
| 41.64 | trans-Caryophyllene | 96 | 13006985 | 1.75 |
| 42.25 | α-Bergamotene | 89 | 2917201 | 0.39 |
| 43.58 | γ-Humulene | 88 | 7018481 | 0.94 |
| 45.41 | α-Longipinene | 90 | 2871425 | 0.39 |
| 46.17 | α-Gurjunene | 89 | 1033281 | 0.14 |
| 46.65 | β-Himachalene | 92 | 3663233 | 0.49 |
| 46.78 | α-Muurolene | 92 | 4123372 | 0.55 |
| 48.15 | α-Copaene | 86 | 2296807 | 0.31 |
| 48.53 | Hydroxy neoisolongifolene | 77 | 1486208 | 0.20 |

(*continues*)

**Table 1.** Continued.

| RT (min) | Compound Name | Match (%) | Area | % Area |
|---|---|---|---|---|
| 52.72 | Isolongifolanon | 87 | 767411 | 0.10 |
| 53.44 | Isolongifolanone | 85 | 7958220 | 1.07 |
| 56.96 | (–)-Isolodene | 80 | 790141 | 0.11 |
| 57.47 | Veridiflorol | 82 | 912271 | 0.12 |
| 58.11 | β-Guaiene | 81 | 621861 | 0.08 |

(n = 3), and the %RSD values for major compounds were below 2%, indicating excellent method repeatability. These compounds were categorized into major classes such as monoterpenes, sesquiterpenes, terpene alcohols, esters, and oxygenated terpenoids.

Among them, sesquiterpenes were the dominant class, accounting for 71.3% of the total normalized peak area. The most abundant compound was isolongifolene (30.07%), followed by isobornyl acetate (15.87%), borneol (12.29%), and (+)-longicyclene (12.44%).

The prominence of isolongifolene aligns with previous studies that highlight the dominance of sesquiterpenes in pyrolyzed pine tar extracts (Shinozaki et al., 2022). These compounds are well-documented for their anti-inflammatory, antimicrobial, and insecticidal activities, underscoring their biotechnological potential (Ali et al., 2015).

Monoterpenes such as α-pinene, β-pinene, and camphene were detected in trace amounts (<0.1%), likely due to their lower thermal stability or high volatility. In contrast, oxygenated terpenoids like borneol (12.29%), d-fenchyl alcohol (0.17%), and isofenchol (0.41%) exhibited higher extraction efficiency, highlighting the capability of DLLME to recover polar volatiles effectively.

Isobornyl acetate and borneol are particularly notable for their antiseptic, anti-inflammatory, and aromatherapeutic properties (Ali et al., 2015). Their substantial presence further pine tar as a valuable feedstock for pharmaceutical intermediates and eco-conscious perfumery formulations. The identification of hydroxylated sesquiterpenes such as veridiflorol and elemol supports the potential for biocatalytic derivatization through enzyme-mediated processes.

Additionally, rare and diagnostic compounds like tetramethyl azulene (2.27%) and γ-himachalene (0.49%) were identified. These markers have been identified as indicators of pyrolytic degradation of lignin and resin acids in several studies, serving as chemical indicators of destructive distillation in Pinus species. These markers have been identified as indicators of pyrolytic degradation of lignin and resin acids in several studies, serving as chemical indicators of destructive distillation in Pinus species (Simoneit et al., 1993).

Normalization of peak areas enabled a reasonable estimate of compound distribution. This profile (Table 1) can serve as a reference for the standardization and authentication of commercial pine tar products. Moreover, the pattern supports further exploration of microbial synthesis pathways for high yield sesquiterpenes such as isolongifolene.

In conclusion, this study not only provides a comprehensive volatile profile of pine tar but also establishes a methodological and conceptual framework for its valorization in bioeconomy.

Future studies should focus on full quantification using isotopically labeled standards and evaluation of seasonal and species-specific variability in pine tar volatiles. Additionally, integration with metabolomics platforms (e.g., GC×GC-MS, LC-QTOF) could provide deeper insights into the less abundant yet biologically relevant metabolites. The coupling of DLLME with online derivatization and automation also offers exciting avenues for process intensification in natural product analytics.

In terms of green chemistry, the DLLME-GC-MS platform demonstrates significant sustainability advantages. The reduced solvent usage (especially chlorinated solvents) and low sample input align with the 12 principles of green analytical chemistry. This positions the method as a scalable and eco-conscious tool for both research and industrial settings. Furthermore, the entire process—from extraction to GC-MS analysis—can be completed in under 2 hours, offering rapid turnaround for commercial screening.

Moreover, the detection of minor azulene-based markers such as tetramethyl azulene suggests potential use in traceability and authentication studies. Azulenes possess unique chromophoric properties, making them useful in analytical fingerprinting and UV–vis-based detection. Their presence also provides indirect evidence of the pyrolytic origin of the pine tar and can serve as thermal degradation markers in quality control protocols.

The presence of oxygenated terpenoids such as borneol and isoborneol highlights potential downstream applications in pharmaceutical formulations. Borneol derivatives are widely studied for their CNS-modulating, analgesic, and anti-inflammatory effects, and their semisynthetic analogs are employed in traditional Chinese medicine and transdermal drug delivery systems. Enzymatic derivatization of these alcohols using oxidoreductases or acetyltransferases offers a green route to functionalized derivatives with improved solubility and bioavailability.

The identification of high-abundance sesquiterpenes such as isolongifolene, (+)-longicyclene, and isobornyl acetate underscores the potential of pine tar as a valuable renewable source for biotechnological exploitation. Isolongifolene, which constituted over 30% of the total peak area, is particularly attractive due to its tricyclic framework, which is amenable to microbial biosynthesis and bioengineering. Recent advances in synthetic biology have demonstrated the feasibility of producing sesquiterpene scaffolds through engineered *Escherichia coli* and *Saccharomyces cerevisiae* strains, using terpene synthase genes and mevalonate pathway optimization (Liu et al., 2022).

### *Cytotoxic effect of pine tar*

In the current study, the effect of pine tar on HUVEC cell viability was evaluated, and the results are given in Figure 1. A significant decrease in cell viability was observed only at the two highest concentrations ($p < 0.0001$); cell viability decreased to ~4% at 1250 µg/mL and ~74% at 625 µg/mL, while there was no decrease in cell viability at the other concentrations. The $IC_{50}$ value of pine tar was calculated as 825 ± 1.8 µg/mL. There is limited information in the literature regarding the cytotoxic effect of pine tar. Dalkilic et al. (2024) investigated the cytotoxic effects of pine tar extracts, prepared with methanol and chloroform, on human breast adenocarcinoma cells (MCF-7 and MDA-MB-231) (Dalkilic et al., 2024). The pine tar extracts exhibited high cytotoxicity depending on the dose. In another study, pine tar showed cytotoxic activity with an IC50 value of 132.9 µg/mL on human cervical adenocarcinoma (HeLa) cells. On the other hand, no cytotoxic effect was observed in non-cancerous mouse fibroblast (L929) cells (Özgen, 2025). To the best of our knowledge, this is the first study to evaluate the cytotoxic effects of pine tar on HUVEC.

## Conclusions

The present study successfully applied DLLME coupled with GC-MS to characterize the volatile composition of pine tar. A total of 45 compounds were identified with match scores ≥77%,

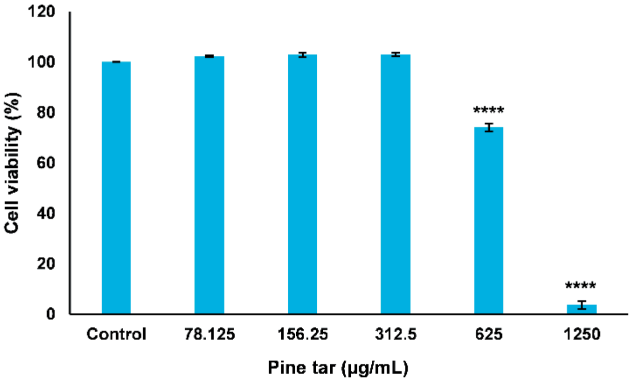

**Figure 1.** Cytotoxic effect of pine tar on HUVEC cells after 24 h of exposure. ***p < 0.0001 vs. control.

confirming the reliability of the method. Sesquiterpenes, particularly isolongifolene, emerged as the dominant chemical class, accounting for over 70% of the total peak area. This composition aligns with previous studies on pyrolyzed pine derivatives and underscores the biological relevance of sesquiterpenes due to their antimicrobial, anti-inflammatory, and insecticidal properties. The analytical approach offers multiple advantages, including rapid extraction, reduced solvent use, and high enrichment efficiency—highlighting its suitability as a green alternative to traditional techniques such as hydrodistillation. The chemical profile generated can serve as a reference for standardization and quality control of pine tar-based products. Moreover, the findings suggest promising biotechnological applications, including the microbial synthesis of high value terpenoids and the development of eco-friendly pharmaceuticals and aroma compounds. Along with this, the low cytotoxicity of pine tar suggests a favorable preliminary safety profile and supports its potential for further biological evaluation.

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## Conflict of interest

The authors declare no conflict of interest.

## Data availability statement

Data can be obtained from the corresponding author upon a reasonable request.

## Ethics committee approval

Ethics committee approval is not required for this study.

## Authors' contribution statement

The authors acknowledge their contributions to this paper as follows: Study conception and design: O.S., A.K.; Data collection: M.N., S.A.; Analysis and interpretation of results: M.N, S.A., GAU; Manuscript draft preparation: A.K. All authors reviewed the results and approved the final version of the manuscript.

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