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RESEARCH ARTICLE OPEN ACCESS

Detection of Antibacterial Components from Chloroform and Ethanolic Extracts of *Flacourtia indica* (Burm. F) Merr. by TLC-Bioautography-based GC-MS and ^{1}H NMR Profiling

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Abstract

***Flacourtia indica* (Burm. F). Merr., a plant widely used in ethnomedicine, was investigated for its antibacterial properties and chemical composition using preparative thin-layer chromatography (TLC) bioautography, gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy. Bioautography was performed on silica gel-coated TLC plates against *Streptococcus pneumoniae*, *Vibrio cholerae*, and *Escherichia coli*. The ethanolic extract exhibited a clear inhibition zone against *E. coli*, while the chloroform extract showed significant inhibition against *S. pneumoniae*. GC-MS analysis revealed 48 compounds in the ethanolic extract and 20 in the chloroform extract. Major compounds identified in the chloroform fraction included sulfurous acid hexyl nonyl ester, phenol derivatives, phthalic acid esters, siloxanes, heneicosane, and piperidinone derivatives. To further confirm the bioactive constituents, ^{1}H NMR spectroscopy was performed on bands exhibiting antibacterial activity. The analysis indicated the presence of aliphatic acyclic, β-substituted aliphatic, and α-monosubstituted aliphatic compounds. These findings suggest that the chloroform extract of *F. indica* contains several aliphatic group chemicals with notable antibacterial activity. The study highlights the potential of isolating, purifying, and characterizing these compounds for pharmaceutical applications, reinforcing the ethnomedicinal relevance of *F. indica* as a source of bioactive molecules.**

Keywords: *Streptococcus pneumoniae, Vibrio cholerae,* TLC-Bioautography, Gas Chromatography-mass Spectrometry (GC-MS), Nuclear Magnetic Resonance (^{1}H-NMR)

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INTRODUCTION

Since ancient times, the biological activity of plant extracts have been evaluated. Medicines made from plants have greatly enhanced human health and wellbeing. The demand for novel antimicrobials that can successfully combat drug-resistant microorganisms has skyrocketed.[1] Secondary metabolites are mostly responsible for the antibacterial qualities of plant materials.[1,2] The primary classes of plant antimicrobial compounds include phenols and polyphenols (flavonoids, quinones, tannins, coumarins, etc.), terpenoids, alkaloids, saponins, steroids, lectins, fatty acids, and polypeptides.[1] The purpose of the current study was to investigate *Flacourtia indica* a medicinal plant for phytochemical and antibacterial properties.

Flacourtia indica, the governor's plum or Indian plum, is a member of the Flacourtiaceae family. A bushy shrub with spiky branches, it has been recognized for its medicinal properties and has been reviewed as an endemic vascular plant limited to the Eastern Ghats of India, namely Odisha.[3] It is utilized as indigenous medicine for a number of illnesses and has a lengthy folklore history. It has been discovered that the phytochemical-rich plant *F. indica* possesses a number of biological qualities, such as antibacterial, antifungal, antioxidant, and anticancer effects.[4] Its antibacterial, antifungal, antioxidant, antimalarial, anti-diabetic, analgesic, anti-inflammatory, and antipyretic properties have all been validated in studies to demonstrate its effectiveness against human infections. The analgesic, anti-inflammatory, and antipyretic effects of *Flacourtia indica's* ethanolic extract have also been investigated.[5] Forty-one different secondary metabolites with antibacterial, antifungal, antioxidant, anti-cancer, and anti-proliferative qualities have been identified in *Flacourtia indica* root extract. According to Al Bashera et al.[6] these extracts have also demonstrated anti-proliferative efficacy against a lung cancer cell line. Thirteen phenolic glycosides and fourteen additional compounds were discovered from the methanolic extract of *Flacourtia indica* leaves, twelve of which were composed of different oxidized forms of pyrocatechuic acid.[7] For more research on the possible health advantages of *F. indica*, it is imperative to examine these bioactive components.

Crude ethanol and chloroform extracts of *F. indica* have shown strong antibacterial activity against a variety of bacterial strains, as demonstrated in previous investigations by our researchers. The antibacterial activity of the chromatographic separated antimicrobial potential of tested plant species has not been evaluated using TLC bioautographic techniques, despite the fact that they are commonly used for evaluating the antimicrobial potential of plant materials, according to a thorough review of the literature on this plant. In order to investigate the phytochemical contents of ethanol and chloroform extracts of *F. indica* which showed notable antibacterial action, the TLC bioautography technique was applied to a test bacterial strain, followed by GC-MS and ^{1}H NMR spectroscopy.

In the current investigation, a few unique bioactive components were discovered from the aerial portions of *F. indica*. Keeping in view the plant extract's separation in TLC-IB and demonstration of antibacterial activity, ^{1}H NMR spectroscopy was used to get exact structural information on the constituent chemicals.

MATERIALS AND METHODS

Plant material and extract preparation

Plant materials were collected, identified, and prepared in accordance with procedure.[8] Dr. P.C. Panda, the lead scientist at the Regional Plant Resource Centre (RPRC), Bhubaneswar, identified and validated fresh *Flacourtia indica* samples that were obtained from the Regional Plant Resource Centre. The plant's fresh stem and leaves were rinsed with tap water and then distilled water, let to air dry for 15 days in the shade, then cut into small pieces and processed into a coarse powder using an electric grinder before being placed in airtight bottles for further analysis. Ethanol and chloroform were used as solvents for a 72 hour Soxhlet extraction of the coarse powder at 30 °C. Following filtering, a rotary vacuum evaporator (BUCHI TYPE, CEMC/RS/01) was used to concentrate the liquid extract, which was then kept in a sterile bottle at 4 °C for further examination.

Bacterial strains

The Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, provided the microbial cultures used in the experiment. The study employed Gram-positive bacteria, *Streptococcus pneumoniae* (MTCC 655), Gram-negative bacteria, *Vibrio cholerae* (MTCC 3900) and *Escherichia coli* (MTCC 1687).

TLC-Bioautography of the chromatograms

The process of bioautography is used to isolate the physiologically active ingredient in plant extract. TLC-IB, or thin-layer chromatography-agar overlay, is an experiment used to perform separation and visualization on a TLC barrier. The eluent system ethyl acetate:benzene (1:5) is employed in this study to thin-layer chromatograph ethanolic and chloroform extracts on pre-coated commercial aluminium TLC plates (Merck, silica gel 60 F_{254}). It was decided on a suitable solvent system before the chromatographic separation. Following chromatographic separation, the resulting TLC plates were allowed to thoroughly dry at room temperature for two hours in order to guarantee that all of the solvents had been removed. The bioautography screening of the chromatographically separated fractions of the *F. indica* leaf was performed using conventional techniques. The produced chromatogram is immersed in a molten 1% nutritional agar bacterial solution. For the investigation, bioautography was done using a culture of *Streptococcus pneumoniae, Vibrio cholerae*, and *Escherichia coli,* which had previously demonstrated high sensitivity to the ethanolic and chloroform extracts of the aerial sections of the plant *F. indica*. After solidification, the plates were incubated for 24 hours at room temperature (37 °C) before staining with FCR. TLC-IB is widely used to discover physiologically active substances that may be further spectroscopically analyzed to learn more about their structure.

Gas chromatography and mass spectrometry

The chloroform and ethanolic extract of *F. indica* was subjected to a GC-MS analysis utilizing an Instrument MassHunter GC/MS Acquisition. This gadget operates at 230 °C and uses mass selective detectors and a detector (MS source). The auxiliary temperature (interphase) was 270 °C, while the injector temperature was 240 °C. Using a single quadrupole of version 6.00.34, the quadrupole temperature in this technique was 150 °C. Helium gas was used as the carrier gas with a flow rate of 1.5 ml/min. The split ratio was 100:1, where the mass spectral scan range was 40 to 550 amu, and the ionization voltage used was 70 eV (International). HP-5MS capillary column was used (30 mm length, 0.25 mm in diameter, 0.025 µm in film thickness at temperatures of 50-325 °C/350 °C). The spectra of the unknown component and the known components, which included the compound's name, chemical formula, molecular weight, and structure, were compared using the NIST data library (NISTVer.17.LMS data library). The temperature programming used for the samples had an initial temperature of 80 °C and a hold period of two minutes. At a rate of 4.5 C/min, the temperature was raised to 180 °C, held for 5 minutes, and then increased to 230 °C, for a total run length of 61.5 minutes. The proportional percentage amount of each component is calculated by comparing the average peak area of each component to the total area.

NMR

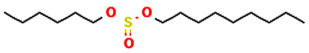
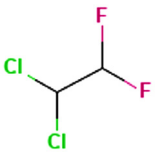
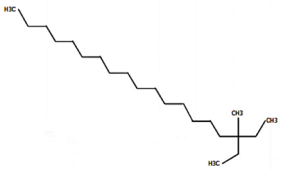
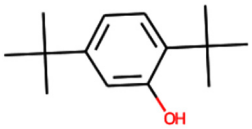
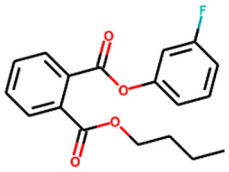
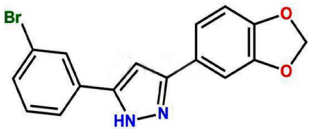
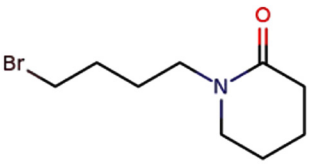
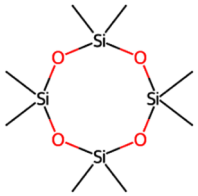
NMR spectra were obtained using a Bruker Avance III 400 spectrometer fitted with a 5 mm multinuclear inverse probehead and running at a frequency of 400.13 MHz for protons. An NMR-400 MHz was employed to record the NMR spectra of the ethanol and chloroform extracts of *F. indica*. Chemical shifts are represented as δ values. Using $CDCl_3$ (deuterated chloroform) as a baseline, the result graph was compared to the reference chart to identify any possible functional groups in the plant.[9]

RESULTS

TLC bioautography assay

The TLC fractionation of the chloroform and ethanolic extracts of *F. indica* revealed the presence of four distinct bands (Figure 1a). The findings revealed high antibacterial characteristics of a fraction. Both the chloroform and ethanolic extracts showed considerable antibacterial action at the point of spotting against Gram-positive bacteria *Streptococcus pneumoniae* (MTCC 655) and Gram-negative bacteria *Vibrio cholerae* (MTCC

Table 1. Major Compounds identified in the chloroform and ethanol extract of *F. indica* using GC-MS

RT	Phytocomponents	Molecular formula	Chemical structure	Chemical Nature	Biological activity
5.749	Sulfurous acid, hexyl nonyl ester	$C_{15}H_{32}O_3S$		Aliphatic	Antioxidants[10,11]
8.000	Ethane, 1,1-dichloro-2,2-difluoro-	$C_2H_2Cl_2F_2$		Aliphatic	Antibacterial[12]
9.104	3-Ethyl-3-methyl-nonadecane	$C_{22}H_{46}$		Aliphatic	Antimicrobial[13]
9.614	Phenol, 2,5-bis (1,1-dimethylethyl)	$C_{14}H_{22}O$		Aromatic	Antioxidant[10,11]
11.177	Phthalic acid, 3-fluoro-phenyl heptadecyl ester	$C_{31}H_{43}FO_4$		Aromatic	Antibacterial, antimicrobial, antiinflammatory, antitumor,[12] antimicrobial, anti-inflammatory, anti-tumor[14]
12.112	Cyclooctasiloxane, hexadecamethyl	$C_{16}H_{48}O_8Si_8$		Aromatic	Antioxidant and antimicrobial[10,11]
13.131	Heneicosane	$C_{21}H_{44}$		Aliphatic	Antimicrobial[12,15,16]
14.287	2-Piperidinone, N-[4-bromo-nbutyl]	$C_9H_{16}BrNO$		Aromatic	Antimicrobial activity[17,18]
18.391	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$		Aromatic	Antioxidant[13]

3900) and *Escherichia coli* (MTCC 1687) (Figure 1b-d). After separation, only one of the four bands remained physiologically active (Figure 1b-d). On TLC plates, light-colored dots against a purple background indicated the presence of antibacterial compounds at ZOI (Figure 2). The development of a zone of inhibition surrounding the band containing the chemical or substances with antibacterial activity served as proof of this activity.

GC-MS analysis

The major compounds with their retention time (RT), molecular formula, and chemical structure from the GC-MS analysis of the chloroform and ethanolic extracts of *F. indica* aerial parts are shown in Table 1. The current study demonstrated that a total of 20 biomolecules were analyzed from the *F. indica* leaf extract using chloroform as a solvent, while 48 biomolecules were analyzed from the ethanolic extract.

NMR

A ^{1}H NMR study of the chloroform extract of *F. indica* revealed various peaks between δ 0-2, δ 2-3 and δ 5-6 (Figure 3), whereas the ethanolic extract showed signals only in the δ 0-2 ppm region (Figure 4). According to the reference charts by Silverstein et al.,[19] the peaks

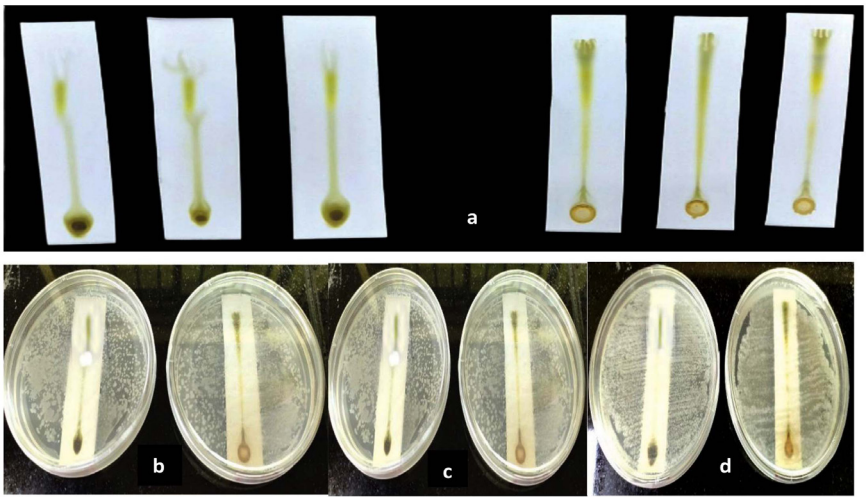


Figure 1. Bioautography Screening: (a) Developed chromatograms of chloroform and ethanolic extract of *F. indica* on TLC silica gel 60 F_{254} plates, Bioautogram screening of chromatograms (TLC-IB) against: (b) *Streptococcus pneumoniae*, (c) *Escherichia coli* (d) *Vibrio cholerae.* Eluent system ethyl acetate: Benzene (1:5)

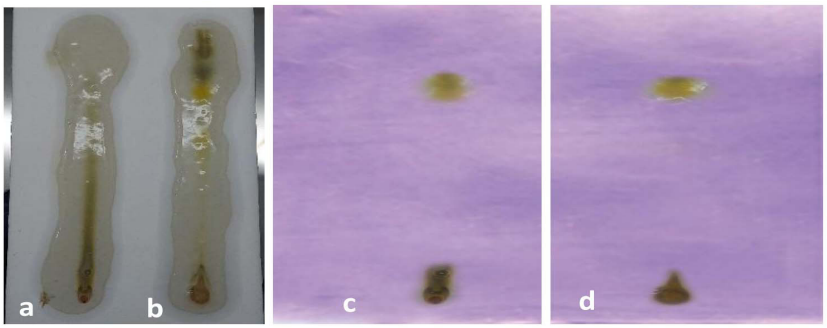


Figure 2. Bioautograms: (a) Developed bioautogram of chloroform extract with 1% molten agar *S. pneumoniae* solution, (b) Developed bioautogram of ethanolic extract with 1% molten agar *E. coli* solution, bioautogram of (c) chloroform and (d) ethanolic extract of *F. indica* after staining with FCR/derivatization with FCR

observed in the chloroform extract correspond to acyclic aliphatic protons, β-substituted aliphatic protons, and α-monosubstituted aliphatic systems (Table 2). A strong, broad signal at δ 1.2-1.5 ppm indicates the presence of methyl (-CH_3) and methylene (-CH_2) groups in long aliphatic chains. Minor peaks between δ 2.0-3.0 ppm can be attributed to methylene groups adjacent to carbonyl functionalities (-CH, -CO-) or protons near ester groups, while protons on carbons adjacent to electronegative atoms (Oxygen, nitrogen or halogens) may also resonate in this region. Additional signals at δ 3.5-3.75 ppm suggest protons in close proximity to oxygen atoms, consistent with alcohol, ether, or ester moieties. A small but distinct peak at δ 5.3-5.4 ppm represents vinylic or deshielded protons adjacent to strong electron-withdrawing groups. The absence of downfield aromatic signals (6-8 ppm), aside from the residual $CDCl_3$ signal at δ~7.26 ppm, indicates

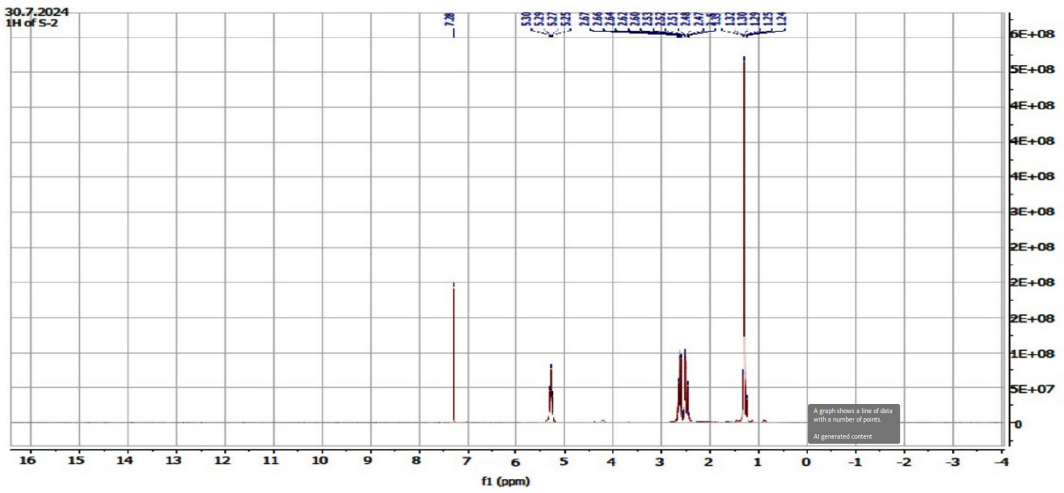


Figure 3. NMR Spectroscopy of chloroform extract of *F. indica.* ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (d, *J* = 6.4 Hz, 7H), 2.47 (dd, *J* = 6 Hz, 2H), 2.61 (dd, *J* = 7.6 Hz, 2H), 5.25 (q, *J* = 6.4 Hz, 2H)

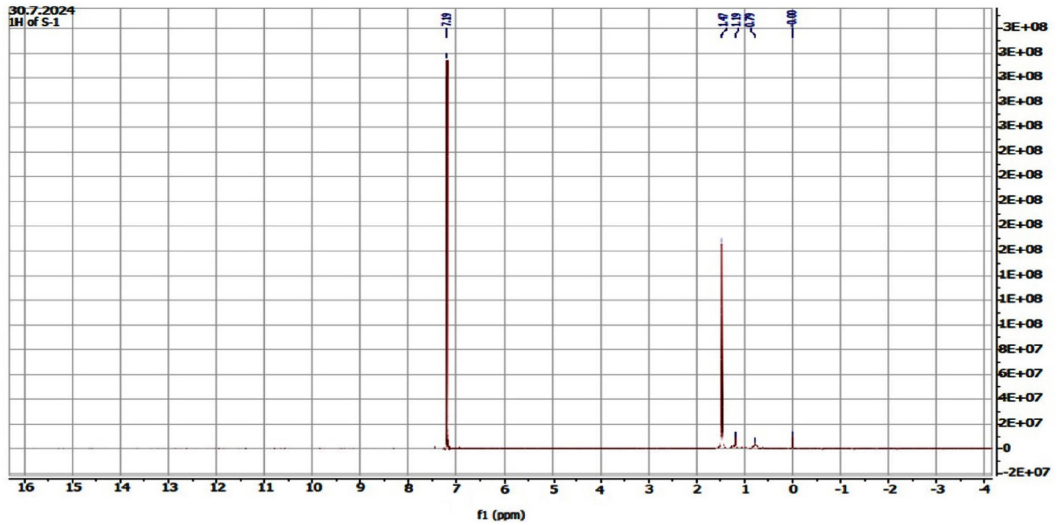
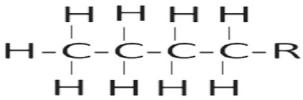
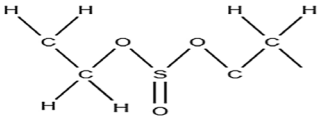
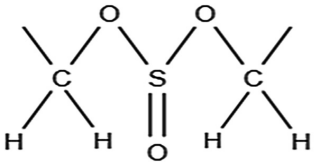


Figure 4. NMR Spectroscopy of ethanolic extract of *F. indica.* ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47 (s, 3H)

Table 2. Potential functional groups and their structures present in the chloroform and ethanolic extract of *F. indica* as analyzed by ^{1}H NMR

Peak in PPM	Possible type of group of compounds	Possible structure
0-2	Aliphatic alicyclic compounds	
2-3	β-Substituted aliphatic compounds	
5-6	α-Mono substituted Aliphatic compounds	

'R' = Elongation of chain; 'X' = Attachment of any functional group

a lack of aromatic ring structures. There may be alcohol groups (-OH), and the peaks around 3.5-4.0 ppm could correspond to protons in proximity to hydroxyl groups. A small but clear peak around 5.3-5.4 ppm represent protons on carbon adjacent to strong electron-withdrawing groups. A small residual peak at δ~7.26 ppm was given by $CDCl_3$. Whereas, in ethanolic extract, δ 1.0-2.2 ppm indicates the presence of methyl (-CH_3) and methylene (-CH_2) groups, revealing the presence of aliphatic alicyclic compounds only (Figure 4). Muharni et al.[20] revealed chemical constituents from the stem bark of Flacourtia rukam, which align with the FTIR-identified functional groups observed in the crude ethanolic and chloroform extracts in the present study.

The spectrum of ^{1}H NMR indicated a doublet at δ 1.27 ((d, *J* = 6.4 Hz, 7H), a doublet doublet at δ 2.47 ((dd, *J* = 6 Hz, 2H), another doublet doublet at δ 2.61 ((dd, *J* = 7.6 Hz, 2H), and a quartet at δ 5.25 (q, *J* = 6.4 Hz, 2H) in the chloroform extract whereas the ethanolic extract indicated a singlet at δ 1.47 (s,3H).

DISCUSSION

The present study successfully employed TLC-bioautography, GC-MS, and ^{1}H NMR spectroscopy to identify and characterize antibacterial constituents in chloroform and ethanolic extracts of *Flacourtia indica*. The bioautography assay revealed clear zones of inhibition against *Streptococcus pneumoniae* and *Escherichia coli*, confirming the presence of bioactive compounds with antimicrobial potential. Notably, the chloroform extract demonstrated stronger activity against *S. pneumoniae*, while the ethanolic extract was more effective against *E. coli*, suggesting differential solubility and specificity of active constituents.

GC-MS analysis identified 20 compounds in the chloroform extract and 48 in the ethanolic extract. Key constituents included sulfurous acid hexyl nonyl ester, heneicosane, 2-piperidinone derivatives, siloxanes, and phenolic esters. These compounds have been previously reported for their antimicrobial, antioxidant, and anti-inflammatory properties.[21,22] The presence of fatty

acid methyl esters and alkaloid derivatives further supports the therapeutic potential of *F. indica*[23,24] as recorded during this investigation.

^{1}H NMR profiling revealed peaks corresponding to aliphatic chains, β-substituted, and α-monosubstituted aliphatic compounds. These structural features are consistent with hydrophobic molecules capable of disrupting bacterial membranes, a mechanism supported by previous studies on plant-derived antimicrobials.[25,26]

The use of TLC-bioautography proved effective for rapid screening and localization of antibacterial activity within complex plant matrices. This method has gained traction in phytochemical research due to its ability to preserve compound integrity while enabling direct visualization of bioactivity.[27,28] When integrated with GC-MS and NMR, it offers a robust analytical framework for natural product discovery.

Recent investigations have emphasized the broad pharmacological spectrum of *F. indica*, including its antioxidant,[29] anti-diabetic,[24] hepatoprotective,[30] and wound-healing properties.[23] These findings align with the current study's identification of multifunctional phytoconstituents. Moreover, trace metal analysis by Koperuncholan and Kulandaivel[31] revealed safe levels of essential elements, supporting the plant's suitability for therapeutic use.

The observed antibacterial activity against both Gram-positive and Gram-negative strains is consistent with earlier reports on *F. indica*'s efficacy against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Vibrio cholerae.*[32] The presence of flavonoids, tannins, and terpenoids confirmed through FTIR and HPTLC fingerprinting further validates the plant's antimicrobial profile.[33]

CONCLUSION

This study underscores the antibacterial potential of chloroform and ethanolic extracts of *Flacourtia indica*, through TLC-bioautography enabled direct visualization of active zones, while GC-MS and ^{1}H NMR profiling revealed a diverse array of bioactive compounds, including phenolic esters, aliphatic derivatives and siloxanes.

It supports the ethnomedicinal relevance of *F. indica* and highlights its promise as a source of plant-based antibacterial agents. Future research should focus on isolating individual constituents, validating their pharmacological effects, and exploring compound-target interactions through metabolomics and molecular docking approaches.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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