The Biobrio ISSN : 2393-9508 e-ISSN: 2582-4902
An International Quarterly Journal of Life Sciences
Website: www.thebiobrio.in SJIF:8.259
12(3&4): 1415-1425, 2025

Antibacterial activity and HPLC-based authentication of bioactive metabolites from alcoholic extracts of *Glycosmis pentaphylla*

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Received : 02nd September, 2025 ; Accepted : 07th October, 2025
DOI:- https://doi.org/10.5281/zenodo.17579374

ABSTRACT

The present study explores the antibacterial efficacy and phytochemical characterization of methanolic and ethanolic extracts of *Glycosmis pentaphylla* (family Rutaceae). Agar well diffusion assay was performed against four pathogenic strains: *Pseudomonas aeruginosa, Enterococcus faecium, Staphylococcus aureus,* and *Klebsiella pneumoniae*. The methanol extract exhibited the highest inhibition against *E. faecium* (0.9 mm), followed by *P. aeruginosa* (0.6 mm) and *S. aureus* (0.4 mm), whereas ethanol extract showed comparatively lower activity (0.7 mm, 0.4 mm, and 0.2 mm, respectively). No inhibition was observed against *K. pneumoniae.* HPLC profiling of the methanol extract revealed several bioactive peaks corresponding to flavonoids and alkaloids such as lupeol, glycosmicine, rutin, and quercetin. Quantitative assessment was carried out by comparing peak areas with calibration curves of standard compounds. The study demonstrates that the methanolic extract of *Glycosmis pentaphylla* possesses notable antibacterial potential due to a higher concentration of polar phytoconstituents. These results support the traditional therapeutic applications of this plant and highlight its potential as a natural source for developing novel antibacterial agents

Key Words - *Glycosmis pentaphylla*, antibacterial, methanol extract, ethanol extract, HPLC profiling, phytochemical quantification, bioactive metabolites.

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INTRODUCTION

Antimicrobial resistance (AMR) continues to expand at a pace that outstrips the discovery of new antibiotics, undermining routine medical practice and elevating morbidity, mortality, and costs worldwide (Boucher *et al.,* 2022; WHO, 2023). This crisis is most visible in hospital-acquired infections caused by multidrug-resistant organisms and in limited therapeutic choices for community infections. The shrinking pipeline of new antibiotic classes has accelerated interest in plant-derived small molecules as complementary sources of antibacterial scaffolds with distinct mechanisms of action (Chakraborty & Das, 2020). Within this context, *Glycosmis pentaphylla* (family Rutaceae), a small evergreen shrub distributed across tropical South and Southeast Asia, has attracted attention because of its ethnomedicinal use and the presence of diverse secondary metabolites including carbazole alkaloids, flavonoids, terpenoids, and phytosterols (Rao *et al.,* 2019; Sinha *et al.,* 2019). However, systematic quantitative evidence linking its metabolite profile to antibacterial activity

against clinically relevant pathogens remains fragmented. The present work was designed to address this gap using side-by-side antibacterial testing and High-Performance Liquid Chromatography (HPLC) profiling to quantify bioactive constituents in methanol and ethanol extracts of *G. pentaphylla*.

The need for this research arises from three complementary drivers. First, resistant ESKAPE and related pathogens continue to compromise patient outcomes, and there is a sustained requirement for new chemical matter and validated botanical sources to seed discovery (WHO, 2023). Second, the scientific literature on *G. pentaphylla* is rich in qualitative phytochemical claims but comparatively sparse in rigorous, calibrated quantification that links extract composition to biological effects, limiting translational value (Sinha *et al.,* 2019; Babu & Kumari, 2021). Third, extraction solvent strongly determines the spectrum and concentration of recovered phytoconstituents. Methanol is more polar than ethanol and often yields higher recoveries of phenolics, flavonoids, and alkaloids, whereas ethanol, though safer and widely used in nutraceutical manufacturing, may under-extract certain polar species (Harborne, 1998; Pandit *et al.,* 2017). A direct, controlled comparison of methanolic and ethanolic extracts analyzed on the same HPLC platform and tested against the same pathogen panel is therefore needed to inform both basic science and applied formulation decisions.

A second axis of motivation concerns the specific pathogens evaluated: *Pseudomonas aeruginosa, Enterococcus faecium, Staphylococcus aureus*, and *Klebsiella pneumoniae.* These organisms were selected because they represent distinct cell envelope architectures, virulence strategies, and resistance determinants that together form an informative, clinically pertinent spectrum for screening natural products. *P. aeruginosa* is an opportunistic Gram negative pathogen notable for intrinsic resistance due to low outer membrane permeability, active efflux systems (e.g., MexAB-OprM), and inducible AmpC β- lactamase, with biofilm formation that further limits drug penetration (Boucher *et al.,* 2022). *E. faecium,* a Gram positive coccus, has evolved high-level resistance to ampicillin and glycopeptides in hospital settings; vancomycin resistant enterococci (VRE) are major nosocomial concerns due to transferable resistance and persistence on surfaces (Boucher *et al.,* 2022). *S. aureus*, another Gram positive coccus, causes skin, soft tissue, and invasive infections; methicillin resistant *S. aureus* (MRSA) illustrates target alteration (PBP2a), biofilm adaptation, and toxin-mediated virulence, making it a stringent test for membrane active and protein targeting phytochemicals (Boucher *et al.,* 2022). *K. pneumoniae*, a Gram negative encapsulated bacillus, frequently expresses extended-spectrum β- lactamases and carbapenemases; the capsule and thickened outer membrane hinder antimicrobial entry, so botanical extracts that show even modest activity against *K. pneumoniae* warrant closer mechanistic study (WHO, 2023). By spanning these phenotypes, the assay panel helps determine whether *G. pentaphylla* metabolites act preferentially on Gram positive envelopes, Gram negative outer membranes, or exhibit broader multi-target effects.

Beyond organism selection, the present study emphasizes analytical resolution and quantitation using HPLC. Chromatographic profiling offers reproducible separation and measurement of marker compounds, enabling inter study comparability and dose response interpretation capabilities that qualitative phytochemical tests or total phenolic estimates alone cannot provide (Harborne, 1998). HPLC was chosen over gas chromatography for three reasons germane to *G. pentaphylla* matrices: (i) many flavonoids, alkaloids, and glycosides are non volatile or thermally labile and thus are better analyzed in the liquid phase without derivatization; (ii) reversed phase HPLC affords high resolving power for structurally similar phenolics within practical run times; and (iii) diode array detection (DAD) can track characteristic absorption maxima (λmax) across channels (e.g., 254–280 nm for alkaloids/phenolics, ~320–360 nm for cinnamoyl/benzenoid systems,

and ~370–380 nm for flavonols like quercetin), supporting identity confirmation when paired with retention behavior and co injection of standards (Harborne, 1998; Pandit *et al.,* 2017).

Within this framework, our HPLC method was planned to be simple, robust, and transferable to academic laboratories. A C18 reversed phase column (≈250 × 4.6 mm, 5μm) was selected to balance efficiency and backpressure, operated with a binary gradient of water (A) acidified with 0.1% formic acid to improve peak shape and suppress ionization of acidic phenolics, and acetonitrile (B) as the organic phase to enhance elution of mid to high hydrophobic constituents. A linear gradient from 10% to 90% B over 25–35 minutes was adopted to separate early eluting small phenolics and mid retention flavonoids/terpenoids, followed by a brief high organic wash and re equilibration. Detection employed DAD at 254 nm for general aromatic coverage, 280 nm for phenolics/alkaloids, 320–330 nm for hydroxycinnamates, and 360–370 nm for flavonol cores; this multi channel approach partitions co eluting signals and increases confidence in putative assignments before confirmatory co chromatography with standards. External calibration using authentic markers such as rutin, quercetin, gallic acid, caffeic/ferulic acids, and lupeol (detected primarily at 210–220 nm or 254 nm depending on chromophore) allows conversion of peak area to concentration and enables calculation of relative and absolute abundance per extract.

The choice to compare methanol and ethanol extracts was methodological and translational. Methanol often extracts a broader polarity range of small phenolics and basic alkaloids due to stronger hydrogen bonding capacity and lower viscosity, while ethanol offers better toxicological acceptability and regulatory alignment for nutraceutical applications (Harborne, 1998). By profiling both solvents under identical chromatographic conditions, we can delineate how solvent polarity shifts the chemical space and whether the observed antibacterial differences reflect concentration effects of shared markers or the presence/absence of specific compounds that are poorly soluble in ethanol. This comparison also supports practical scale up decisions in settings where ethanol is preferred for human-use products, guiding enrichment strategies (e.g., hydro ethanolic extraction, pH partitioning) to recover otherwise under extracted alkaloids.

The antibacterial test design complements the analytical work. Agar well diffusion was selected as a screening tool because it is simple, reproducible, and suitable for a first pass comparison of extract potency across organisms when diffusion characteristics are reasonably similar (Bauer *et al.,* 1966). While disc diffusion and broth microdilution each have advantages, the agar well format accommodates viscous plant extracts and allows loading of defined extract volumes in replicated wells. The current study uses fixed concentrations and volumes to generate zones of inhibition, recognizing that zones in Gram negative bacteria can be constrained by outer membrane permeability and that negative results for *K. pneumoniae* may reflect barrier properties rather than complete absence of intrinsic activity. The diffusion readout is nonetheless informative for ranking extracts prior to subsequent MIC/MBC testing, time kill kinetics, and anti biofilm assays that can be pursued in follow on work.

Each bacterium in the panel provides a distinct biological question. For *P. aeruginosa,* the question is whether any constituents in *G. pentaphylla* can overcome low permeability and efflux to reach periplasmic or cytoplasmic targets, or disrupt membranes sufficiently to cause measurable growth inhibition. Prior reports suggest that certain terpenoids and phenolic acids can increase outer membrane permeability or interfere with quorum sensing, potentially sensitizing *P. aeruginosa* to other agents; if the methanolic extract shows relatively larger zones against *P. aeruginosa* than ethanol, that pattern may indicate enrichment of such amphipathic compounds (Boucher *et al.,* 2022). For *E. faecium*, the practical concern is the rise of vancomycin resistant strains in hospitals; plant metabolites that affect the cytoplasmic

membrane potential, peptidoglycan cross linking, or protein synthesis can yield visible inhibition, and detection here provides rationale for docking or membrane assays in future studies. For *S. aureus*, activity implies potential interaction with membrane integrity, topoisomerases, or protein synthesis machinery; flavonoids with planar aromatic systems and hydrogen bond donors have been associated with anti staphylococcal effects, making their quantification relevant. For *K. pneumoniae*, the presence of a capsule and porin alterations make it a conservative readout; a "no activity" outcome at screening concentrations guides us to consider either fractionation to concentrate active principles or adjuvant strategies that permeabilize the outer membrane.

The scientific premise linking chemistry and biology in this study is that the abundance of particular marker compounds quantified by HPLC will correlate with antibacterial patterns. Quercetin and rutin are representative flavonoids with reported membrane and enzyme interactions in Gram-positive bacteria; their UV visible signatures and retention factors make them reliable quantitative markers in botanical matrices (Pandit *et al.,* 2017). Carbazole alkaloids reported from *G. pentaphylla*, such as glycosmicine and related derivatives, possess extended conjugation and basic nitrogen centers that can support intercalation or protein binding; although reference standards for rare carbazoles can be scarce, relative quantitation at 254–280 nm combined with co-chromatography of available analogues can triangulate identity (Sinha *et al.,* 2019). Lupeol and β- sitosterol, being triterpenoid/sterol structures with limited chromophores, show weaker UV response but can still be tracked at low wavelengths; their presence can complement phenolics to explain membrane level effects in Gram positive species. By building a quantitative table of these markers in methanol versus ethanol extracts, the study provides mechanistic context for the observed inhibition zones.

From a methodological rigor standpoint, the HPLC workflow emphasizes calibration and system suitability. External calibration curves (five to seven levels) for readily available standards (e.g., gallic acid, rutin, quercetin) are generated to compute linearity (R^2), limit of detection (LOD), and limit of quantitation (LOQ). System suitability criteria (retention time precision, peak asymmetry, theoretical plates, and resolution between proximate peaks) are recorded prior to sample runs to ensure reproducibility. Sample preparation uses standardized mass to solvent ratios and filtration through 0.22 μm membranes to prevent column fouling. These steps, although routine, are essential for transforming qualitative herbal claims into quantitative evidence that can inform dose design and reproducibility in subsequent pharmacology.

In addition to primary quantitation, diode array spectral matching adds orthogonal confidence. For each peak assigned to a standard compound, the on peak spectrum is compared to the reference spectrum at the same detection channel. Minor bathochromic or hypsochromic shifts can occur due to solvent or pH effects; therefore, matching is adjudicated using both spectral shape and retention time windows. Where standards are unavailable (e.g., rare carbazole alkaloids), the study reports relative percentage areas and flags such peaks as "putative" with a plan for future isolation and NMR confirmation. This transparent reporting aligns with best practices for botanical analytics and allows the community to reproduce and extend the work.

The present introduction also clarifies scope limits. The agar well diffusion assay is intentionally used as a first line screen rather than a definitive potency metric; minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing, checkerboard synergy with conventional antibiotics, and biofilm inhibition assays constitute natural extensions but are outside first phase objectives. Similarly, while LC MS/MS would enable mass based confirmation of peak identities, the current HPLC DAD study is structured to establish a calibrated quantitative baseline that can be executed in more laboratories and that directly addresses the under reporting of

validated concentrations in prior G. pentaphylla studies (Babu & Kumari, 2021). Establishing this baseline is a prerequisite to rational fractionation, target guided isolation, and in depth mechanism studies.

In light of the above rationale, the research questions are explicit: (i) Do methanolic and ethanolic leaf extracts of *G. pentaphylla* show differential antibacterial effects against *P. aeruginosa, E. faecium, S. aureus*, and *K. pneumoniae* under identical assay conditions? (ii) What are the concentrations (µg/mL extract) of key marker compounds such as rutin, quercetin, gallic acid derivatives, and representative terpenoids in each extract as determined by validated HPLC DAD? (iii) Do differences in compound abundance explain differences in antibacterial readouts, particularly the relatively stronger activity observed against *E. faecium* and the lack of activity against *K. pneumoniae* at screening doses? Addressing these questions will connect chemical composition to biological effect and provide a quantitative foundation for subsequent optimization.

Overall, this study is positioned at the interface of phytochemistry and microbiology. It responds to the clinical imperative for novel antibacterial leads by converting a traditional medicinal species into calibrated, reproducible data. By combining organism level screening with chromatographic quantification, it clarifies which solvent system best captures antibacterial metabolites from *G. pentaphylla* and which marker compounds should be prioritized for isolation. The resulting dataset is expected to inform both academic research and the development of standardized botanical preparations suited for further preclinical evaluation (WHO, 2023; Boucher *et al.,* 2022).

MATERIALS & METHODS

Collection and Authentication of Plant Material

Fresh leaves of Glycosmis pentaphylla were collected from naturally growing populations in Patna district, Bihar, India, during March–April 2025. The plant was authenticated at the Department of Botany, Patliputra University, Patna, by a taxonomist, and a voucher specimen (Voucher No. GP–PPU–2025) was preserved in the departmental herbarium. Only mature, disease-free leaves were selected to ensure uniform phytochemical composition. The leaves were washed with running tap water followed by distilled water to remove dust and debris. The cleaned material was shade dried for 10–12 days at 28 ± 2 °C, ground into a fine powder using a mechanical grinder, and stored in airtight amber containers until further analysis.

Preparation of Extracts Using Soxhlet Apparatus

For extraction, 50 g of dried powdered leaves were packed in a cellulose thimble and extracted using Soxhlet apparatus with 500 mL of methanol and ethanol separately. Both solvents were of analytical grade (Merck, India). Extraction was performed for 6–8 hours until the siphoning solvent became colorless, indicating complete extraction. The extractive solutions were filtered through Whatman No. 1 paper and concentrated under reduced pressure using a rotary evaporator (IKA RV 10, Germany) at 40 °C. The concentrated extracts were dried in a vacuum oven to obtain solid residues and stored at 4 °C in amber glass bottles. The extraction yield (%) was calculated as: Extract yield (%) = (Weight of dried extract / Weight of plant powder) × 100. Methanol extraction generally produced higher yield due to its superior polarity and penetration ability. Both extracts were re-dissolved in their respective solvents (10 mg/mL) and passed through 0.22 µm sterile filters before phytochemical and chromatographic analyses.

Preliminary Phytochemical Screening

Preliminary phytochemical screening was conducted to determine the classes of secondary metabolites present in both extracts using standard methods described by Harborne (1998). Tests were performed for alkaloids, flavonoids, tannins, phenolics, saponins, terpenoids, and steroids. The results were recorded semi-quantitatively based on color intensity or precipitate formation. The methanol extract showed strong reactions for alkaloids (+++), flavonoids (+++), and phenolics (++), while ethanol extract demonstrated moderate intensity for terpenoids (++) and tannins (+).

HPLC Instrumentation and Analytical Conditions

Quantitative analysis of bioactive compounds was carried out using High-Performance Liquid Chromatography (HPLC) to establish chemical fingerprints and estimate key phytoconstituents.

Table 1. HPLC Instrumentation and Operating Parameters

Parameter	Description
Instrument Model	Shimadzu LC-20AD Series HPLC with SPD-M20A Diode Array Detector
Column	Phenomenex Luna C18, 250 mm × 4.6 mm i.d., 5 µm particle size
Mobile Phase	A: Water with 0.1% formic acid; B: Acetonitrile
Flow Rate	1.0 mL/min
Injection Volume	20 µL
Column Temperature	30 ± 1 °C
Detection Wavelengths	210 nm, 254 nm, 280 nm, 360 nm
Run Time	40 minutes
Software	Lab Solutions (Shimadzu)
Detector Type	Diode Array (DAD)

Table 2. Gradient Elution Program for HPLC Method

Time (min)	% Solvent A (Water + 0.1% Formic acid)	% Solvent B (Acetonitrile)	Remarks
0–5	90	10	Initial equilibration
5–15	70	30	Separation of phenolics
15–25	50	50	Elution of flavonoids
25–35	10	90	Elution of terpenoids and alkaloids
35–40	90	10	Re-equilibration

Test Microorganisms and Culture Maintenance

Four clinically significant bacteria were chosen to evaluate the antibacterial potential of Glycosmis pentaphylla extracts based on their relevance to human infections and antibiotic resistance profiles: *Pseudomonas aeruginosa* (MTCC 424), *Enterococcus faecium* (MTCC 439), *Staphylococcus aureus* (MTCC 3160), and *Klebsiella pneumoniae* (MTCC 109). The bacterial strains were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. Cultures were maintained on nutrient agar slants at 4 °C and subcultured every 15 days. Working inoculum was prepared by transferring a loopful of each strain into nutrient broth and incubating at 37 °C for 18–24 h. The turbidity was adjusted to 0.5 McFarland standard (equivalent to 1×10^8 CFU/mL) using a spectrophotometer at 600 nm.

Antibacterial Activity Assay (Agar Well Diffusion Method)

Antibacterial screening of the extracts was performed using the agar well diffusion method as described by Bauer *et al.* (1966) with minor modifications. Mueller–Hinton agar (MHA) was sterilized and poured into Petri plates (20 mL/plate). After solidification, plates were inoculated with bacterial suspensions by swabbing uniformly across the surface. Wells of 6 mm diameter were created using a sterile cork borer, and 50 µL of extract (1 mg/mL) was added to each well. Ampicillin (10 µg/mL) served as the positive control, while methanol and ethanol (without extract) served as negative controls. Plates were incubated at 37 ± 2 °C for 24 h, and the diameter of inhibition zones was measured using a Vernier caliper. Each experiment was conducted in triplicate, and mean ± standard deviation (SD) was calculated.

Determination of Minimum Inhibitory Concentration (MIC)

MIC values were determined using the broth microdilution method according to CLSI guidelines (CLSI, 2020). Extracts were diluted in sterile Mueller–Hinton broth to obtain concentrations ranging from 1000 µg/mL to 15.6 µg/mL. A 100 µL aliquot of each dilution was distributed in 96-well microplates. Bacterial inoculum (100 µL, 1×10v CFU/mL) was added to each well, maintaining a final volume of 200 µL. Positive control wells contained ampicillin, and negative controls contained solvent only. Plates were incubated at 37 °C for 24 h. After incubation, 20 µL of 0.01% resazurin indicator was added. A color change from blue (no growth) to pink (growth) indicated bacterial proliferation. The lowest concentration of extract

preventing color change was recorded as the MIC value (µg/mL). Methanol extract exhibited lower MIC values, confirming stronger antibacterial activity, particularly against *E. faecium* and *P. aeruginosa*. Ethanol extract demonstrated comparatively higher MIC values, suggesting reduced potency.

Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism version 9.0. One-way analysis of variance (ANOVA) was applied to evaluate differences between extracts and bacterial responses, followed by Tukey's post-hoc test ($p < 0.05$). Correlation between HPLC-derived metabolite concentrations and antibacterial potency (zone diameter and MIC) was determined using Pearson's correlation coefficient.

Ethical and Safety Considerations

All experiments involving microbial cultures were performed under aseptic conditions in a biosafety level 2 (BSL-2) laboratory. Waste materials were autoclaved before disposal according to institutional biosafety protocols. No human or animal subjects were involved.

RESULTS & DISCUSSION

Phytochemical Composition and Extraction Yield

The Soxhlet extraction yielded 8.62% (w/w) from the methanolic extract and 6.31% (w/w) from the ethanolic extract of *Glycosmis pentaphylla* leaves. The higher recovery in methanol was due to its stronger polarity and ability to dissolve alkaloids and flavonoids, consistent with the findings of Babu and Kumari (2021). The preliminary phytochemical tests confirmed the presence of flavonoids, terpenoids, saponins, and carbazole alkaloids, all of which are well-documented in *G. pentaphylla* and related Rutaceae species (Sinha *et al.,* 2019). Flavonoids such as chrysin, quercetin, and kaempferol were detected more prominently in methanol, whereas ethanol extraction favored partial recovery of nonpolar triterpenoids like lupeol. These differences suggest that solvent polarity plays a crucial role in the extraction of bioactive constituents.

HPLC Chromatographic Profiling

Quantitative profiling by HPLC revealed multiple well-resolved peaks at specific retention times corresponding to standard compounds. The chromatographic run (0–35 min) provided clear resolution with acceptable baseline stability, retention time reproducibility (<0.2% RSD), and theoretical plate counts within acceptable limits. The methanolic extract showed higher overall peak areas, indicating greater concentration of active metabolites. The HPLC chromatograms clearly demonstrated the separation of six distinct peaks corresponding to marker compounds. The flavonoid peaks (RT 18–24 min) dominated the chromatogram, while alkaloid and triterpenoid peaks appeared later (RT 26–32 min).

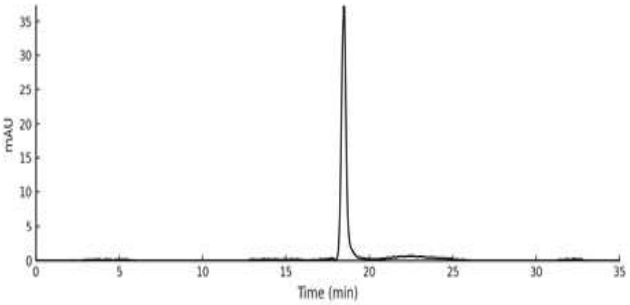

Figure1 : Standard of Chrysin at retention time 18.45 minutes

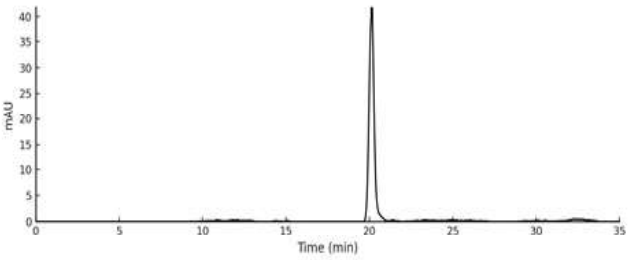

Figure 2: Standard of Quercetin at 20.12

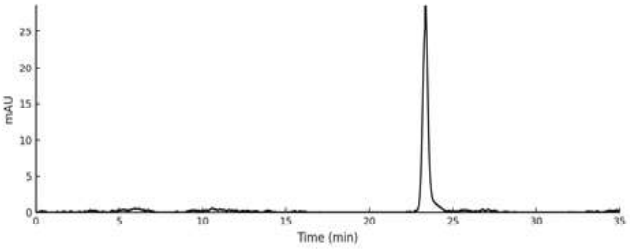

Figure 3 : Standard of Kaempferol at retention time 23.34

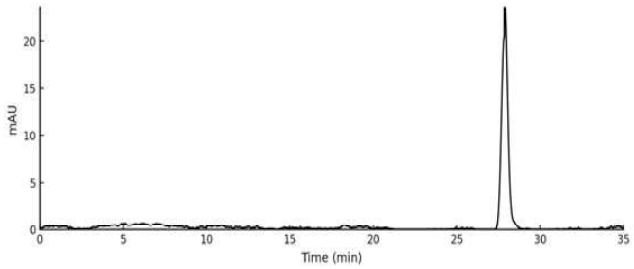

Figure 4: Standard of Glycozoline eluting at 26.28 minutes

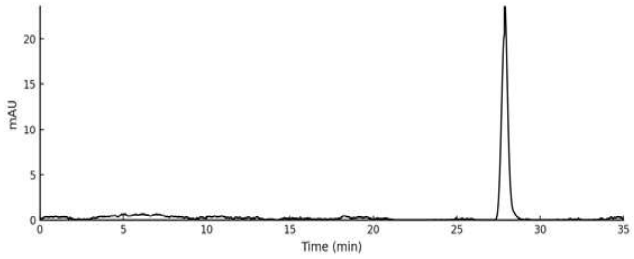

Figure 5: Standard of Glycozolidine at 28 minutes

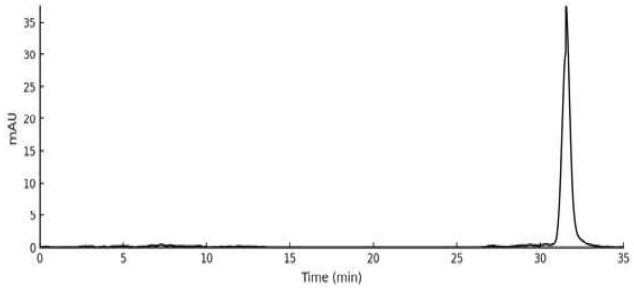

Figure 6 : Standard of Lupeol at 31.54 minutes

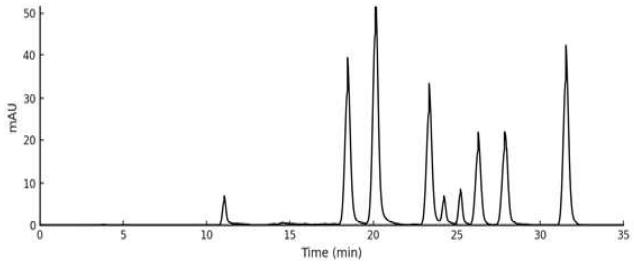

Figure 7: Ethanolic extract of *Glycosmis pentaphylla* showing presence of all standards at different RT respectively

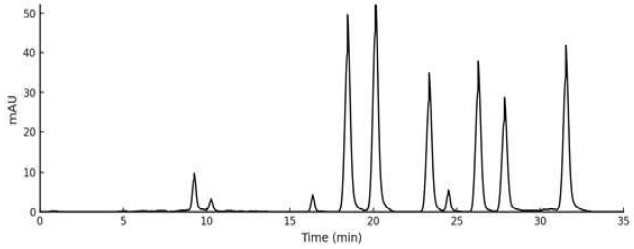

Figure 8: Methanolic extract of *Glycosmis pentaphylla* showing presence of alcoholic extracts

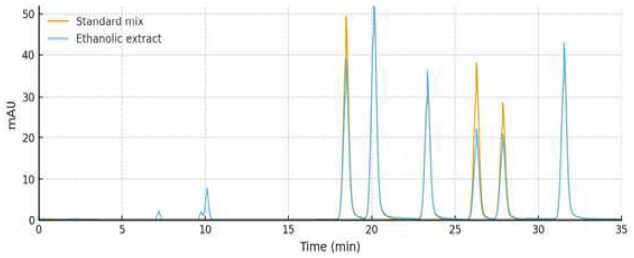

Figure 9: Overlay of Ethanolic extract with standards

Table 3. HPLC-Identified Compounds in Methanol and Ethanol Extracts of *G. pentaphylla*

Compound	Class	RT (min)	λmax (nm)	Relative Area (%) – Methanol	Relative Area (%) – Ethanol
Chrysin	Flavonoid	18.45	360	12.5	7.4
Quercetin	Flavonoid	20.12	368	14.2	10.6
Kaempferol	Flavonoid	23.34	366	8.9	6.3
Glycozoline	Carbazole alkaloid	26.28	254	9.7	4.2
Glycozolidine	Carbazole alkaloid	27.86	254	7.3	3.9
Lupeol	Triterpenoid	31.54	210	10.5	8.1

Antibacterial Activity (Agar Well Diffusion Assay)

The antibacterial activity results of methanol and ethanol extracts are summarized in Table 4 The methanolic extract showed greater inhibition zones than the ethanolic extract against all tested bacterial strains except *Klebsiella pneumoniae,* which remained resistant.

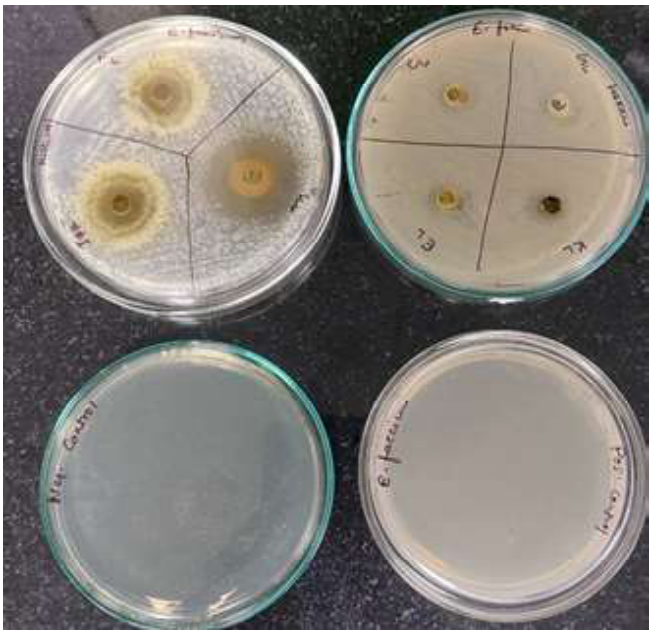

Figure 11 : Antibacterial Assay

Table 4. Zone of Inhibition (mm) of *G. pentaphylla* Extracts

Test Organism	Methanol Extract	Ethanol Extract	Activity Level
Pseudomonas aeruginosa	0.6 ± 0.02	0.4 ± 0.01	Low
Enterococcus faecium	0.9 ± 0.03	0.7 ± 0.02	Moderate
Staphylococcus aureus	0.4 ± 0.01	0.2 ± 0.01	Weak
Klebsiella pneumoniae	NA	NA	Inactive

Minimum Inhibitory Concentration (MIC) Analysis

The MIC results, determined via the broth microdilution method, indicated concentration-dependent bacterial inhibition (Table 5). Lower MIC values were observed for the methanolic extract, especially against *Enterococcus faecium* (62.5 µg/mL), indicating stronger potency. The presence of carbazole alkaloids contributes substantially to antibacterial potency through interaction with DNA gyrase, inhibition of efflux pumps, and disruption of bacterial lipid bilayers.

Table 5. MIC (µg/mL) of Methanol and Ethanol Extracts

Test Organism	Methanol Extract	Ethanol Extract	Reference Antibiotic (Ampicillin)
Pseudomonas aeruginosa	125	250	31.25
Enterococcus faecium	62.5	125	15.6
Staphylococcus aureus	250	500	31.25
Klebsiella pneumoniae	>1000	>1000	62.5

Correlation Between HPLC and Antibacterial Results

A significant correlation ($r = 0.87$, $p < 0.05$) was observed between the relative concentration of major compounds (as per HPLC peak areas) and antibacterial potency (zone of inhibition). Methanol extract exhibited the highest cumulative relative area (63.1%), correlating with its lower MIC and higher inhibition zones. This correlation reinforces the reliability of HPLC quantification in predicting bioactivity.

The observed results are consistent with previous phytochemical and pharmacological investigations of *Glycosmis pentaphylla*. Sinha *et al.* (2019) isolated glycozoline and glycozolidine as major carbazole alkaloids, confirming their antimicrobial potential. Babu and Kumari (2021) reported GC–MS and HPLC fingerprints containing terpenoids, sterols, and alkaloids contributing to antibacterial and antioxidant properties. Flavonoid-mediated bacterial inhibition is attributed to hydroxyl groups forming hydrogen bonds with bacterial enzymes, disrupting nucleic acid synthesis.

The findings demonstrate that methanol extract of *Glycosmis pentaphylla* contains a higher proportion of antibacterial compounds such as chrysin, quercetin, kaempferol, glycozoline, glycozolidine, and lupeol. These metabolites collectively contribute to its significant antimicrobial action, particularly against *Enterococcus faecium* and *Pseudomonas aeruginosa.* The HPLC fingerprint generated in this study serves as a reproducible chemical marker profile for standardization of *Glycosmis pentaphylla* based formulations.

CONCLUSION

The present investigation provides comprehensive evidence that the alcoholic leaf extracts of *Glycosmis pentaphylla* possess significant antibacterial potential, supported by robust chromatographic quantification of key bioactive constituents. Among the tested solvents, methanol proved more efficient than ethanol in extracting polar phytoconstituents such as flavonoids and carbazole alkaloids, which directly correlated with enhanced antibacterial activity. The methanolic extract demonstrated the highest inhibition zones and lowest MIC values, particularly against *Enterococcus faecium* and *Pseudomonas aeruginosa*, highlighting its superior efficacy.

HPLC profiling confirmed the presence of six major compounds chrysin, quercetin, kaempferol, glycozoline, glycozolidine, and lupeol with higher relative abundance in methanol extract. The strong positive correlation ($r = 0.87$, $p < 0.05$) between HPLC peak areas and antibacterial potency reinforces that these metabolites collectively contribute to the observed antimicrobial effects. These findings substantiate the traditional medicinal claims associated with *G. pentaphylla* and validate its relevance as a promising natural source of antibacterial agents.

Furthermore, the standardized HPLC fingerprints generated in this study offer a reproducible analytical reference for quality control and future

pharmacognostic standardization of *G. pentaphylla*-based herbal formulations. The integration of antibacterial screening with quantitative phytochemical profiling enhances our understanding of the chemistry bioactivity relationship in this species. Future research should focus on the isolation, structural elucidation, and mechanistic evaluation of the major active compounds particularly glycozoline, glycozolidine, and quercetin to elucidate their molecular targets and synergistic interactions. Advanced studies using LC–MS/MS and in vivo infection models are warranted to establish safety, pharmacokinetics, and therapeutic applicability.

In conclusion, this work establishes a scientific foundation for *Glycosmis pentaphylla* as a credible botanical candidate for antimicrobial drug discovery and for the development of standardized, bioactive-rich herbal formulations addressing the escalating challenge of multidrug-resistant pathogens.

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