



Preliminary phytochemical profiling, antibacterial activity and cytotoxic evaluation of *Simarouba glauca* (DC.) leaves

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Abstract

Medicinal plants remain a productive source of bioactive molecules for antimicrobial drug discovery. This study compared the phytochemical composition and antibacterial activity of aqueous, methanolic, chloroform and hexane leaf extracts of *Simarouba glauca* DC. and assessed the cytotoxicity of the methanolic extract towards mammalian cells. Extracts were prepared by Soxhlet extraction and screened qualitatively for the major classes of secondary metabolites. Antibacterial activity was determined against *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli* and *Pseudomonas aeruginosa* by the agar well diffusion method. Cytotoxicity of the methanolic extract in L929 mouse fibroblasts was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Alkaloids, carbohydrates and terpenoids were detected in all four extracts, whereas flavonoids, phenolic compounds and tannins were largely confined to the aqueous and methanolic extracts. The aqueous extract produced the largest zone of inhibition, 20.33 ± 0.58 mm against *S. aureus*. Two-way analysis of variance confirmed a significant effect of extraction solvent on inhibition for every organism tested ($p < 0.001$), the aqueous extract exceeding the methanolic, chloroform and hexane extracts in all pairwise comparisons; the hexane extract was inactive throughout. Fibroblast viability remained above 70 % at 100 $\mu\text{g/mL}$ of the methanolic extract and the IC_{50} of 155.1 $\mu\text{g/mL}$ is an extrapolated value, since 50 % inhibition was not reached within the range tested. Under ISO 10993-5 criteria the extract is therefore classified as non-cytotoxic. The combination of measurable antibacterial activity in the polar extracts with low toxicity towards mammalian fibroblasts identifies the aqueous and methanolic leaf extracts of *S. glauca* as suitable starting material for bioassay-guided fractionation and for the development of plant-derived topical and oral-care antibacterial formulations.

Keywords: agar well diffusion; cytotoxicity; ISO 10993-5; L929 fibroblasts; secondary metabolites; Simaroubaceae; solvent polarity

Received: 06 July 2026. **Accepted:** 03 September 2026. **Published:** Version 1.0: 29 September 2026; Version 2.0: 01 October 2026.

Cite this article: Kundekar PS, Hegde HV, Manoj ML, Jigan SI, Chougule A, Jadhav A. Preliminary phytochemical profiling, antibacterial activity and cytotoxic evaluation of *Simarouba glauca* (DC.) leaves. Plant Science Today. 2026; 13(4): 1-16. <https://doi.org/10.14719/pst.16591>

1. Introduction

Traditional healthcare systems have depended significantly on medicinal plants, which continue to be valuable sources of new therapeutic compounds (1). In developing countries approximately 80 % of the population depends on plant-based medicine for primary health care (2). The demand for alternative antimicrobial agents derived from natural sources has intensified with the spread of multidrug-resistant bacteria (3, 4). Phytochemicals derived from medicinal plants have attracted attention because of their wide range of biological activities, including antibacterial, antioxidant, anti-inflammatory and anticancer effects (5). By targeting microbial cell walls, membranes and metabolic pathways,

phytochemicals such as alkaloids, flavonoids, tannins, glycosides and terpenoids are known to exert strong antibacterial activity (6–8). The choice of extraction solvent determines both the range and the yield of phytoconstituents recovered, since each solvent selectively dissolves compounds within a particular polarity window (9). Aqueous and methanolic solvents, in particular, have been widely reported to recover compounds with potent antimicrobial activity (10).

The antibacterial potential of *Simarouba glauca* DC. leaf extracts is already established. Antimicrobial activity alongside qualitative phytochemical profiles, broad-spectrum antimicrobial, antioxidant and anticancer activity *in vitro*; and

anthelmintic and anticoagulant effects have been reported for *S. glauca* leaf phytochemicals (11–14).

Three gaps nonetheless remain. First, these studies have generally characterised one or two extracts, so the relationship between solvent polarity and antibacterial activity in this species has not been examined systematically under uniform conditions; because polarity governs which metabolite classes are recovered, this relationship determines the choice of solvent for any subsequent fractionation. Second, antibacterial and cytotoxicity endpoints have rarely been measured on the same extract within a single experimental series, yet it is the relationship between them, rather than either value alone, that determines whether an extract merits development. Third, the activity of *S. glauca* against oral pathogens has received little attention, although *Streptococcus mutans* is the principal aetiological agent of dental caries and an oral-care application would require precisely the combination of antibacterial activity and fibroblast biocompatibility that a screen of this design can establish.

In order to determine the safety profile and potential therapeutic applicability of plant-derived bioactive phytochemicals, cytotoxicity assessment plays a pivotal role (15). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is one of the most reliable colorimetric assays commonly employed to assess cell viability and cytotoxic effects (16). In the present study the cytotoxicity of the methanolic leaf extract was assessed in L929 murine fibroblasts by the MTT assay. The methanolic extract was selected based on its rich phytochemical composition and broad-spectrum antibacterial activity observed during preliminary screening (11, 17).

The present study therefore compares aqueous, methanolic, chloroform and hexane Soxhlet extracts of *S. glauca* leaves, prepared from a single batch of material and evaluated against a common panel of two Gram-positive and two Gram-negative pathogens under identical conditions and assesses the cytotoxicity of the most promising extract against L929 murine fibroblasts using ISO 10993-5 criteria. We hypothesised that antibacterial activity would track the recovery of polar secondary metabolites across the solvent series and that the extract showing the broadest antibacterial spectrum would prove non-cytotoxic at the concentrations at which that activity was observed. The specific objectives were: (i) to compare the phytochemical composition of the four extracts by qualitative screening; (ii) to determine and compare their antibacterial activity by agar well diffusion against *Staphylococcus aureus*, *S. mutans*, *Escherichia coli* and *Pseudomonas aeruginosa* and (iii) to evaluate the cytotoxicity of the methanolic extract towards L929 fibroblasts by MTT assay and to classify it against ISO 10993-5.

2. Materials and Methods

Study site and plant material

The study was carried out at the Indian Council of Medical Research–National Institute of Traditional Medicine (ICMR-NITM), Belagavi, Karnataka, India, between November 2025 and March 2026.

Fresh healthy leaves of *S. glauca* were collected in December 2025 from the campus of the ICMR-NITM, Belagavi, India (15°51' N, 74°30' E; 752 m above sea level). The site has a tropical climate with a mean annual rainfall of approximately 1468 mm. The specimen was authenticated by a qualified taxonomist and a voucher specimen was deposited in the herbarium of ICMR-NITM, Belagavi, under accession number RMRC-1693. Leaves were transported to the laboratory immediately after collection.

The leaves were washed thoroughly with distilled water to remove surface dirt and adhering particles, shade-dried at room temperature and ground to a fine powder in a laboratory mixer grinder. The powdered material was stored in an airtight container at room temperature until extraction.

Preparation of leaf extracts

Four separate 20 g portions of dried leaf powder were extracted in a Soxhlet apparatus with 250 mL of distilled water, methanol, chloroform or hexane. Extraction times were 8 hr for water, 7 hr for methanol and 6 hr each for chloroform and hexane, in every case continued until the siphoning solvent ran colourless. Each extract was filtered through Whatman No. 1 filter paper.

Solvent was removed on a water bath maintained at 45 °C inside a fume hood. Evaporation under the forced convection of the fume-hood airflow proceeds by continuous removal of saturated vapour from the liquid surface and does not require the solvent to reach its boiling point, so water and methanol were removed at a bath temperature well below their atmospheric boiling points. A temperature of 45 °C lies below the range of approximately 60–70 °C at which hydrolysis of glycosides, oxidation of catechols to quinones and decarboxylation of phenolic acids become appreciable and no extract was exposed to a higher temperature at any stage.

The dried extracts were weighed and the yield calculated on a dry-weight basis as:

Yield (% w/w) = (mass of dried extract / mass of dried leaf powder) × 100

Yields were 24.0 % (aqueous), 33.3 % (methanolic), 20.5 % (chloroform) and 7.5 % (hexane). The dried extracts were stored in airtight containers at 4 °C until use.

The extracts were not standardised against a chemical marker and are characterised here only by extraction solvent and yield. Standardisation to total



phenolic content or to a marker constituent identified chromatographically is a prerequisite for reproducible dosing and is identified below as necessary further work.

Phytochemical screening

For qualitative screening, each dried extract was redissolved in its own extraction solvent at 10 mg/mL and filtered through Whatman No. 1 filter paper. This filtered preparation is referred to throughout as the test solution and the same preparation was used for every reagent test. All four extracts were screened in parallel by every test and each test was performed in triplicate.

The tests depend on characteristic colour or precipitation reactions: alkaloids form insoluble complexes with heavy-metal iodides; carbohydrates undergo acid-catalysed dehydration to furfural derivatives that condense with α -naphthol or reduce cupric to cuprous ion; phenolics and tannins form coloured complexes with Fe^{3+} and precipitate with lead acetate; flavonoids give chalcone salts in alkali; and terpenoids give coloured products on dehydration in concentrated sulfuric acid.

Detection of alkaloids

Alkaloids were screened by Mayer's and Wagner's tests, performed as described previously (17, 18).

Mayer's test: To 2 mL of test solution, 1–2 drops of Mayer's reagent were added along the side of the tube. A creamy white to yellow precipitate indicated alkaloids.

Wagner's test: To 2 mL of test solution, 1–2 drops of Wagner's reagent were added along the side of the tube. A brown to reddish precipitate indicated alkaloids.

Detection of carbohydrates

Carbohydrates were screened by Molisch's, Benedict's, Fehling's and iodine tests, performed as described previously (17, 18).

Molisch's test: To 2 mL of test solution, 2–3 drops of Molisch's reagent were added, followed by 1–2 mL of concentrated sulphuric acid (H_2SO_4) along the side of the tube. A purple or violet ring at the interface indicated carbohydrates.

Benedict's test: To 2 mL of test solution, 2 mL of Benedict's reagent was added and the mixture heated in a boiling water bath for 2–5 min. A greenish-yellow precipitate indicated reducing sugars.

Fehling's test: Equal volumes of Fehling's A and B were added to 2 mL of test solution and the mixture heated in a water bath. A brick-red precipitate indicated reducing sugars.

Iodine test: To 2 mL of test solution, 2–3 drops of iodine solution were added. A blue-black colour indicated starch.

Detection of glycosides

Glycosides were screened by the Keller–Killiani, Borntrager's, modified Borntrager's and Raymond's tests, performed as described previously (17, 18).

Keller–Killiani test: To 2 mL of test solution, 1.5 mL of glacial acetic acid and 1–2 drops of FeCl_3 were added, followed by concentrated H_2SO_4 along the side of the tube. A brown ring at the interface with a bluish-green upper layer indicated cardiac glycosides.

Borntrager's test: 2 mL of test solution was treated with dilute H_2SO_4 and boiled, cooled, extracted with chloroform and ammonia added to the chloroform layer. A red colour in the ammoniacal layer indicated anthraquinone glycosides.

Modified Borntrager's test: The test solution was boiled with ferric chloride (FeCl_3) and hydrochloric acid (HCl), cooled and extracted with chloroform; ammonia was added to the chloroform layer. A pink to red colour indicated C-glycosides.

Raymond's test: A few millilitres of test solution were treated with dinitrobenzene in hot methanolic alkali. A red colour indicated cardiac glycosides.

Detection of proteins

Proteins and amino acids were screened by the biuret, ninhydrin, xanthoproteic and Millon's tests, performed as described previously (18, 19).

Biuret test: To 2 mL of test solution, 1 mL of 10 % Sodium hydroxide (NaOH) was added, followed by 2–3 drops of 0.5 % Copper sulphate (CuSO_4). A violet or purple colour indicated proteins or peptide bonds.

Ninhydrin test: To 2 mL of test solution, 2–3 drops of ninhydrin reagent were added and the mixture heated in a boiling water bath for 1–2 min. A blue or purple colour indicated amino acids and proteins.

Xanthoproteic test: To 2 mL of test solution, 1 mL of concentrated nitric acid (HNO_3) was added; the mixture was heated gently, cooled and excess NaOH or ammonium hydroxide (NH_4OH) added. A yellow colour turning orange indicated aromatic amino acids.

Millon's test: To 2 mL of test solution, Millon's reagent was added and the mixture heated gently. A white precipitate turning red indicated tyrosine-containing proteins.

Detection of flavonoids

Flavonoids were screened by the alkaline reagent and lead acetate tests, performed as described previously (20, 21).

Alkaline reagent test: To 2 mL of test solution, a few drops of NaOH were added. An intense yellow

colour that became colourless on adding dilute acid indicated flavonoids.

Lead acetate test: To 2 mL of test solution, a few drops of lead acetate were added. A yellow precipitate indicated flavonoids.

Detection of phenolic compounds

Phenolic compounds were screened by the ferric chloride, lead acetate and Folin–Ciocalteu tests, performed as described previously (20, 21).

Ferric chloride test: To 2 mL of test solution, 2–3 drops of 5 % FeCl_3 were added. A black coloration indicated phenolic compounds.

Lead acetate test: To 2 mL of test solution, a few drops of lead acetate were added. A bulky precipitate indicated phenolic compounds.

Folin–Ciocalteu test: To 2 mL of test solution, Folin–Ciocalteu reagent was added, followed by Na_2CO_3 . After 10–15 min a blue colouration indicated phenolic compounds.

Detection of tannins

Tannins were screened by the FeCl_3 , lead acetate and potassium dichromate tests, performed as described previously (20, 21).

Ferric chloride test: To 2 mL of test solution, 2–3 drops of 5 % FeCl_3 were added. A greenish-black colour indicated tannins.

Lead acetate test: To 2 mL of test solution, a few drops of lead acetate were added. A bulky precipitate indicated tannins.

Potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) test: To 2 mL of test solution, 1 % $\text{K}_2\text{Cr}_2\text{O}_7$ was added. A brown precipitate indicated tannins.

Detection of saponins

Saponins were screened by the froth, foam and sodium bicarbonate tests, performed as described previously (19, 21).

Froth test: 2 mL of test solution was taken with 5 mL of distilled water, shaken vigorously for 30 sec and allowed to stand for 10–15 min. A stable froth of ≥ 1 cm indicated saponins.

Foam test: The test solution was shaken vigorously with water and foam stability observed. Persistence of foam for up to 10 min confirmed saponins.

Sodium bicarbonate (NaHCO_3) test: To 2 mL of test solution, a few millilitres of sodium bicarbonate solution and a few millilitres of distilled water were added and the mixture shaken vigorously. A stable honeycomb-like froth indicated saponins.

Detection of terpenoids

Terpenoids were screened by the Liebermann–Burchard, Salkowski and copper acetate tests, performed as described previously (17, 18).

Liebermann–Burchard test: To 2 mL of test solution, acetic anhydride was added, followed by a few drops of concentrated H_2SO_4 . A reddish colour indicated terpenoids.

Salkowski test: To 2 mL of test solution, 2 mL of concentrated H_2SO_4 was added along the side of the tube. A reddish-brown coloration indicated terpenoids.

Copper acetate test: To 2 mL of test solution, 3–4 drops of copper acetate were added. An emerald-green colour indicated diterpenes.

Detection of gums and mucilages

Gums and mucilages were screened by the alcohol precipitation test, performed as described previously (18, 19).

Alcohol precipitation test: To 2 mL of test solution, excess absolute alcohol was added. Turbidity indicated gums and mucilage.

Detection of resins

Resins were screened by the acetone–water and sulfuric acid tests, performed as described previously (18, 19).

Acetone–water test: The test solution was treated with acetone and distilled water added. A precipitate indicated a positive result.

Sulfuric acid test: A small amount of test solution was treated with concentrated H_2SO_4 . A reddish-brown coloration indicated a positive result.

Detection of oils and fats

Oils and fats were screened by the saponification and spot tests, performed as described previously (18, 19).

Saponification test: To 2 mL of test solution, alcoholic potassium hydroxide (KOH) and a few drops of phenolphthalein were added and the mixture heated in a water bath. Formation of soap indicated oils and fats.

Spot test: A drop of test solution was placed on filter paper and allowed to dry. A permanent translucent spot indicated oils and fats.

Antibacterial assay

Antibacterial activity was evaluated by the agar well diffusion method (22). Sterile Mueller–Hinton agar plates (HiMedia, Mumbai, India) were inoculated with the test organisms *S. aureus* ATCC 29737 and *S. mutans* ATCC 25175 (Gram-positive) and *Escherichia coli* ATCC 4157 and *P. aeruginosa* ATCC 9027 (Gram-negative).

Each dried extract was reconstituted in its own extraction solvent to a stock concentration of 10 mg/mL. Wells of 6 mm diameter were cut in the seeded agar with a sterile cork borer and 25, 50, 75 or 100 μ L of stock was dispensed into separate wells, delivering 0.25, 0.50, 0.75 and 1.00 mg of extract per well respectively.

Streptomycin (HiMedia) served as the positive control and was tested over the same four-step dilution series as the extracts (100, 75, 50 and 25 % of stock) on a separate plate for each organism; the positive control was recorded as a single determination at each level and the values are therefore reported without a standard deviation. An equal volume of methanol, loaded into a single central well, served as the solvent negative control and was run against each of the four organisms on its own plate. Plates were incubated at 35 °C for 24 hr, after which the diameter of the zone of inhibition around each well was measured with a calibrated ruler. All experiments were performed in triplicate on three independent occasions and results expressed as mean $\pm$ standard deviation.

In vitro cytotoxicity assessment by MTT assay

In vitro cytotoxicity of the methanolic extract of *S. glauca* (MSG-01) was evaluated by MTT assay (16, 23). The L929 mouse fibroblast cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India and used between passages 10 and 20. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % foetal bovine serum at 37 °C in a humidified atmosphere of 5 % CO₂.

Cells were seeded into 96-well flat-bottomed microplates at 1×10^4 cells per well in 100 μ L of medium and allowed to attach overnight. The medium was replaced with medium containing the extract at 3.125, 6.25, 12.5, 25, 50 or 100 μ g/mL, prepared from a stock in dimethyl sulfoxide (DMSO). The final DMSO concentration was held constant at 2.5 % (v/v) in every treated well, so that any contribution of the vehicle to the measured response was identical across the concentration series. Untreated cells cultured in medium alone served as the control against which viability was normalised; a vehicle-only control was not included and the implications of this are considered in the Discussion. Plates were incubated for a further 24 hr.

Wells were washed twice with phosphate-buffered saline (PBS), 20 μ L of MTT solution was added to each well and plates were incubated at 37 °C for 4 hr. Formazan crystals were dissolved in 100 μ L of DMSO and absorbance was recorded at 570 nm on a microplate reader. Viability was calculated as:

$$\text{Cell viability (\%)} = (A_{570} \text{ treated} / A_{570} \text{ untreated control}) \times 100$$

The IC₅₀ was estimated by least-squares linear regression of percentage viability on extract concentration ($y = -0.4013x + 112.2$; $R^2 = 0.9391$) and taken as the concentration at which the fitted line crosses 50 % viability. Because 50 % inhibition was not reached within the range tested, the reported value lies outside the experimental range and is an extrapolation.

Statistical analysis

All experiments were performed in triplicate on three independent occasions and results are expressed as mean $\pm$ standard deviation. Zone of inhibition data were analysed by two-way analysis of variance (ANOVA), with extraction solvent and loaded volume as fixed factors, performed separately for each test organism, followed by Tukey's honestly significant difference test for pairwise comparison of solvents. Cell viability data were analysed by one-way ANOVA across the concentration series; because viability was normalised to the untreated control, each treatment group was additionally compared with the control value by one-sample *t*-test with Bonferroni correction for six comparisons. Differences were considered significant at $p < 0.05$. Analyses were performed in Python 3 using the SciPy statistical library.

3. Results

Qualitative phytochemical analysis

The medicinal potential of plant material is attributed largely to a wide range of bioactive secondary metabolites. Preliminary screening of the aqueous, methanolic, chloroform and hexane leaf extracts of *S. glauca* revealed a broad spectrum of phytoconstituents (Table 1).

Alkaloids were detected in all four extracts by Wagner's reagent, although Mayer's reagent gave a negative result with the chloroform extract; where two reagents for the same class disagreed, the class was recorded as present on the basis of the positive test. Carbohydrates were detected in all four extracts by Molisch's and Benedict's tests, Fehling's test being negative in the hexane extract only and the iodine test negative throughout, indicating the absence of starch. Cardiac glycosides were detected in all four extracts by the Keller–Killiani test and, in the aqueous and methanolic extracts, additionally by Raymond's test; anthraquinone glycosides were not detected by Borntrager's test in any extract, while the modified Borntrager's test gave a positive result for C-glycosides in the chloroform extract alone. Proteins were largely absent, with limited positivity in the xanthoproteic and Millon's tests.

Flavonoids and phenolic compounds were detected mainly in the aqueous and methanolic extracts, with weaker detection in the chloroform



extract and none in the hexane extract. Tannins were confined entirely to the aqueous and methanolic extracts, all three tannin reagents giving negative results with both the chloroform and the hexane extracts.

Terpenoids were present in all solvent extracts, irrespective of solvent polarity. Saponins, gums, mucilages, oils and fats were not detected in any extract. Resins were observed predominantly in the aqueous extract and partially in the methanolic extract.

Table 1. Qualitative phytochemical screening of aqueous, methanolic, chloroform and hexane leaf extracts of *Simarouba glauca*

Test	Aqueous	Methanol	Chloroform	Hexane
Alkaloids				
Wagner's test	+	+	+	+
Mayer's test	+	+	–	+
Carbohydrates				
Molisch's test	+	+	+	+
Benedict's test	+	+	+	+
Fehling's test	+	+	+	–
Iodine test	–	–	–	–
Glycosides				
Keller–Killiani test	+	+	+	+
Borntreger's test	–	–	–	–
Modified Borntreger's test	–	–	+	–
Raymond's test	+	+	–	–
Proteins				
Biuret test	–	–	–	–
Ninhydrin test	–	–	–	–
Xanthoproteic test	+	+	+	–
Millon's test	–	+	–	–
Flavonoids				
Alkaline reagent test	+	+	+	–
Lead acetate test	+	+	–	–
Phenolic compounds				
Ferric chloride test	+	+	+	–
Lead acetate test	+	+	+	–
Folin–Ciocalteu test	–	+	–	–
Tannins				
Ferric chloride test	+	+	–	–
Lead acetate test	+	+	–	–
Potassium dichromate test	+	+	–	–

Test	Aqueous	Methanol	Chloroform	Hexane
Saponins				
Froth test	–	–	–	–
Foam test	–	–	–	–
NaHCO ₃ test	–	–	–	–
Terpenoids				
Liebermann–Burchard test	+	+	+	+
Salkowski test	+	+	+	+
Copper acetate test	+	+	+	+
Gums and mucilage				
Alcohol precipitation test	–	–	–	–
Resins				
Acetone–water test	+	–	–	–
Sulfuric acid test	+	+	–	–
Oils and fats				
Saponification test	–	–	–	–
Spot test	–	–	–	–

Legend: (+) positive, (–) negative. All tests were performed in triplicate.

Overall, the aqueous and methanolic extracts showed a richer phytochemical profile than the chloroform and hexane extracts, indicating that the polar solvents recovered a greater diversity of phytoconstituents. The distribution of metabolite classes tracked solvent polarity: the two polar solvents recovered the phenolic, flavonoid and tannin fractions, whereas the non-polar hexane extract retained only terpenoids, carbohydrates, cardiac glycosides and the alkaloid fraction detected by Wagner's reagent. The extraction yields showed the same broad pattern, the polar solvents recovering the greater mass of material (24.0 % and 33.3 % w/w for water and methanol respectively, against 20.5 % and 7.5 % for chloroform and hexane).

Antibacterial activity

The four extracts were tested against two Gram-positive (*S. aureus*, *S. mutans*) and two Gram-negative (*E. coli*, *P. aeruginosa*) strains at four amounts per well (Table 2, Fig. 1i–x).

The streptomycin positive control produced clear, well-defined zones against all four organisms across the full dilution series (Table 3, Fig. 1a–d), the largest being 39 mm against *S. mutans* and the smallest 12 mm against *P. aeruginosa* at the lowest level tested.

Zone diameter fell monotonically with dilution for every organism, confirming that the assay responded to dose as expected. The solvent negative controls produced no measurable inhibition against any organism, confluent growth extending to the rim of the well in every case (Fig. 1 e–h); the zones recorded for the extracts are therefore attributable to the extracted constituents and not to residual solvent.

Two-way ANOVA showed a significant effect of extraction solvent on the diameter of inhibition for every organism tested: *S. aureus* $F(3, 32) = 704.5$, *S. mutans* $F(3, 32) = 366.2$, *E. coli* $F(3, 32) = 192.8$ and *P. aeruginosa* $F(3, 32) = 79.6$, in each case $p < 0.001$. The loaded volume also had a significant effect ($p < 0.001$ for the three most susceptible organisms; $p = 0.004$ for *P. aeruginosa*) and the solvent $\times$ volume interaction was significant throughout ($p \leq 0.002$), indicating that the four extracts did not respond alike to increasing load.

Tukey's test separated all four solvents from one another for every organism ($p < 0.01$ in all 24 pairwise comparisons), the ranking being aqueous > methanolic > chloroform > hexane. Among the Gram-positive organisms, *S. aureus* was the most susceptible, with a maximum zone of 20.33 ± 0.58 mm at 75 μ L, followed by *S. mutans* at 17.33 ± 1.53 mm at 50 μ L. The



chloroform extract was active only at the two lower amounts and the hexane extract produced no inhibition of either Gram-positive strain at any amount tested.

Among the Gram-negative organisms, the aqueous extract again gave the largest zones, $16.00 \pm$

2.08 mm against *E. coli* and 14.33 ± 2.89 mm against *P. aeruginosa*, both at $75 \mu\text{L}$. The methanolic extract was less active and the chloroform extract showed weak or selective inhibition only at the two lower amounts. The hexane extract was inactive against both Gram-negative organisms.

Table 2. Antibacterial activity (zone of inhibition, mm) of *Simarouba glauca* leaf extracts against four bacterial pathogens

Microorganism	Amount per well (mg)	Aqueous	Methanol	Chloroform	Hexane
Gram-positive					
<i>Staphylococcus aureus</i>	1.00	15.33 ± 2.52	10.33 ± 1.00	0	0
	0.75	20.33 ± 0.58	11.33 ± 1.00	0	0
	0.50	18.33 ± 1.53	16.00 ± 0.58	15.00 ± 1.53	0
	0.25	17.67 ± 1.00	15.00 ± 1.15	13.50 ± 0.58	0
	Solvent control	0	0	0	0
<i>Streptococcus mutans</i>	1.00	13.33 ± 2.65	9.00 ± 1.53	0	0
	0.75	16.67 ± 1.73	9.67 ± 1.00	0	0
	0.50	17.33 ± 1.53	14.00 ± 1.53	11.50 ± 1.53	0
	0.25	16.67 ± 1.00	12.67 ± 1.53	12.00 ± 1.15	0
	Solvent control	0	0	0	0
Gram-negative					
<i>Escherichia coli</i>	1.00	11.00 ± 3.79	9.00 ± 2.52	0	0
	0.75	16.00 ± 2.08	8.67 ± 1.15	0	0
	0.50	15.67 ± 2.65	11.67 ± 1.53	7.50 ± 1.00	0
	0.25	15.00 ± 0.58	11.33 ± 1.15	11.50 ± 1.00	0
	Solvent control	0	0	0	0
<i>Pseudomonas aeruginosa</i>	1.00	10.00 ± 3.06	6.67 ± 0.00	0	0
	0.75	14.33 ± 2.89	7.67 ± 1.53	0	0
	0.50	11.33 ± 0.58	10.00 ± 1.00	7.00 ± 1.15	0
	0.25	10.67 ± 0.58	9.33 ± 6.35	7.00 ± 0.58	0
	Solvent control	0	0	0	0

Values are mean $\pm$ SD of three independent experiments and include the 6 mm diameter of the well. Streptomycin (HiMedia) served as the positive control and the corresponding extraction solvent and sterile distilled water as negative controls. 0 = no measurable zone. Two-way ANOVA showed a significant effect of solvent for every organism ($p < 0.001$); Tukey's test separated all four solvents in all pairwise comparisons ($p < 0.01$).

The mean zone diameter was larger for the two Gram-positive organisms (14.60 mm) than for the two Gram-negative organisms (11.15 mm) when the aqueous and methanolic extracts were considered together. With only two organisms in each group, however, this difference

did not reach statistical significance ($p = 0.124$) and the greater susceptibility of the Gram-positive strains is therefore reported as a consistent trend rather than as a demonstrated effect. The activity co-occurred with the polar metabolite classes detected in these extracts—

flavonoids, tannins, phenolics and alkaloids (Table 1)–although the present data establish an association rather than a causal relationship.

Cytotoxicity assessment

The cytotoxicity of the methanolic leaf extract (MSG-01) was evaluated in L929 mouse fibroblasts

by MTT assay over a concentration range of 3.125–100 µg/mL ($n = 3$; Table 4). The methanolic extract was selected because it showed broad-spectrum antibacterial activity across all four organisms and contained a wide range of phytochemical classes.

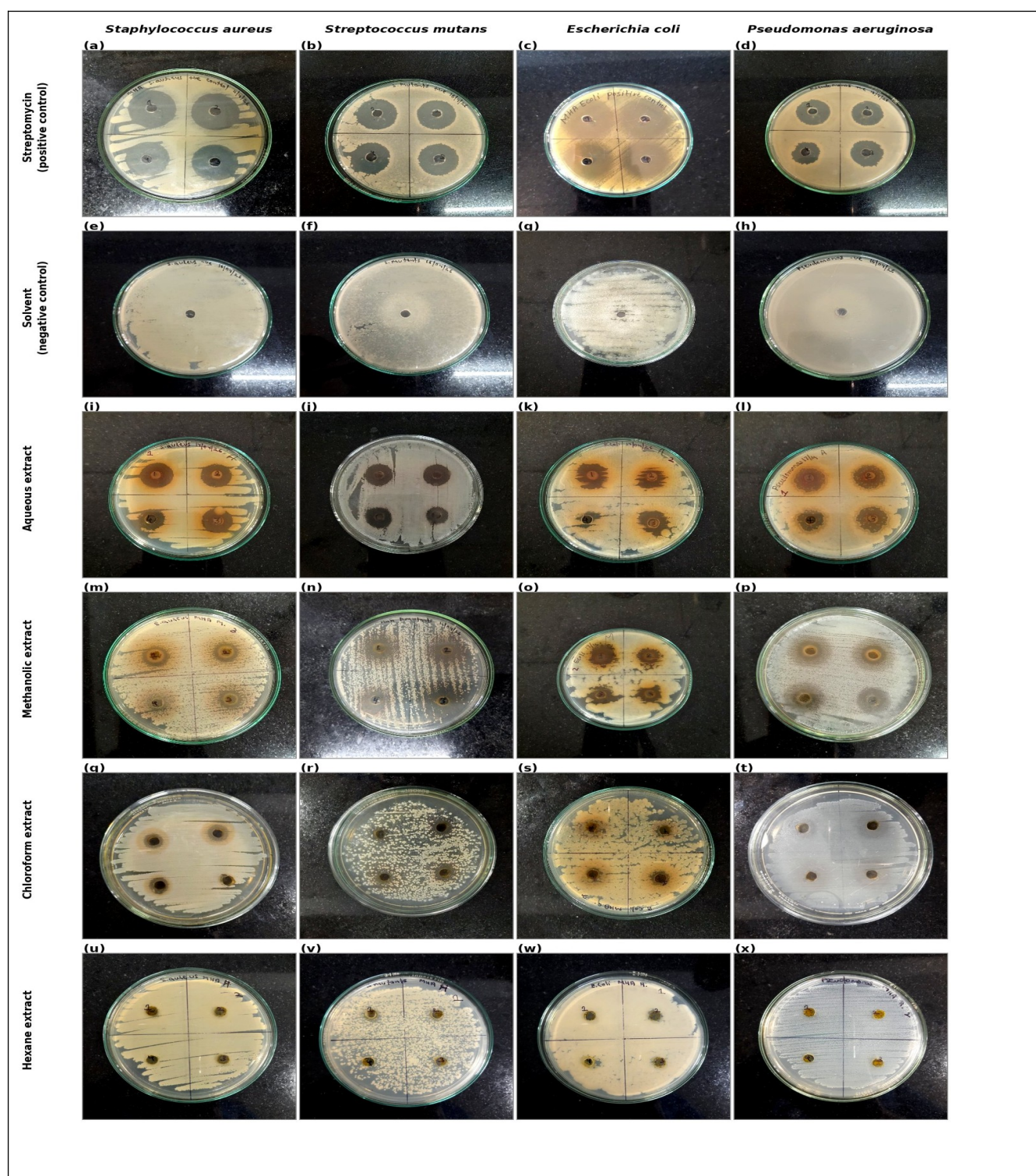


Fig. 1. Agar well diffusion plates for the four test organisms. Columns, left to right: *Staphylococcus aureus* ATCC 29737, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 4157 and *Pseudomonas aeruginosa* ATCC 9027. (a–d) Streptomycin positive control; the four wells correspond to 100, 75, 50 and 25 % of the streptomycin stock and the resulting zone diameters are given in Table 3. (e–h) Solvent negative control, a single central well loaded with the extraction solvent alone; confluent growth

extends to the rim of the well in every case, with no measurable zone of inhibition. (i–l) Aqueous extract, (m–p) methanolic extract, (q–t) chloroform extract and (u–x) hexane extract; in each of these panels the four wells were loaded with 100, 75, 50 and 25 μL of the corresponding 10 mg/mL extract stock, delivering 1.00, 0.75, 0.50 and 0.25 mg per well and the measured zone diameters are given in Table 2. The aqueous and methanolic extracts produced clear zones against all four organisms, the chloroform extract produced smaller zones that were absent against *P. aeruginosa* at the higher loadings and the hexane extract produced no inhibition against any organism. All plates were photographed after 24 hr incubation at 35 °C.

Table 3. Zone of inhibition (mm) produced by the streptomycin positive control against the four test organisms

Streptomycin level	<i>S. aureus</i>	<i>S. mutans</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
100 %	27	39	31	21
75 %	26	37	27	20
50 %	24	36	26	16
25 %	23	33	23	12

Streptomycin (HiMedia) was tested over the same four-step dilution series as the extracts. Values include the 6 mm diameter of the well and are single determinations per level, run on a separate plate for each organism; standard deviations are therefore not available.

Table 4. Viability of L929 mouse fibroblasts after 24 hr exposure to the methanolic leaf extract of *Simarouba glauca* (MSG-01), determined by MTT assay

Sl. No.	Concentration ($\mu\text{g/mL}$)	Mean viability (%)	Standard deviation
1	100	72.88	2.09
2	50	93.40	5.70
3	25	96.89	4.87
4	12.5	105.06	0.85
5	6.25	108.97	3.34
6	3.125	117.00	1.15

Values are mean $\pm$ SD of three independent experiments, expressed as a percentage of the untreated control. One-way ANOVA $F(5,12) = 57.5$, $p < 0.001$. Compared with the untreated control (one-sample t-test, Bonferroni-corrected): 100 $\mu\text{g/mL}$ $p = 0.012$; 3.125 $\mu\text{g/mL}$ $p = 0.009$; 50, 25, 12.5 and 6.25 $\mu\text{g/mL}$ not significant.

Viability differed significantly across the concentration series (one-way ANOVA, $F(5, 12) = 57.5$, $p < 0.001$). A concentration-dependent decrease was observed, the lowest value being 72.88 ± 2.09 % at 100 $\mu\text{g/mL}$, which was significantly below the untreated control ($p = 0.012$ after Bonferroni correction). At 50 and 25 $\mu\text{g/mL}$ viability remained high (93.40 ± 5.70 % and 96.89 ± 4.87 % respectively) and did not differ significantly from the control. At ≤ 12.5 $\mu\text{g/mL}$ viability exceeded that of the untreated control, rising to 117.00 ± 1.15 % at 3.125 $\mu\text{g/mL}$, a significant increase ($p = 0.009$); the full concentration–response profile is shown in Fig. 2.

Viability did not fall below 50 % at any concentration tested. The IC_{50} of approximately 155.1 $\mu\text{g/mL}$ was therefore obtained by extrapolating the fitted line beyond the experimental range plotted in Fig. 2 and should be regarded as an estimate rather than a

measured value. Because viability remained above 70 % throughout, the extract is classified as non-cytotoxic under ISO 10993-5 (24).

4. Discussion

The leaf extracts of *S. glauca* examined here differed markedly in phytochemical composition and in antibacterial activity and the two varied together across the solvent series. The aqueous and methanolic extracts, which recovered the phenolic, flavonoid and tannin fractions, were the active ones; the hexane extract, which did not, was inactive throughout.

Mechanistic basis of the observed activity

The metabolite classes detected in the active extracts have well-characterised and largely distinct antibacterial mechanisms, which may account for the

broad spectrum observed. Flavonoids act at several targets simultaneously: they inhibit bacterial DNA gyrase and topoisomerase IV, perturb the cytoplasmic membrane so as to dissipate the proton motive force and interfere with ATP synthase and nucleic acid synthesis (25). Their potency is structure-

dependent, the number and position of hydroxyl substituents on the B-ring and the presence of the C2–C3 double bond being the principal determinants of membrane partitioning; glycosylation reduces membrane permeation and generally lowers activity.

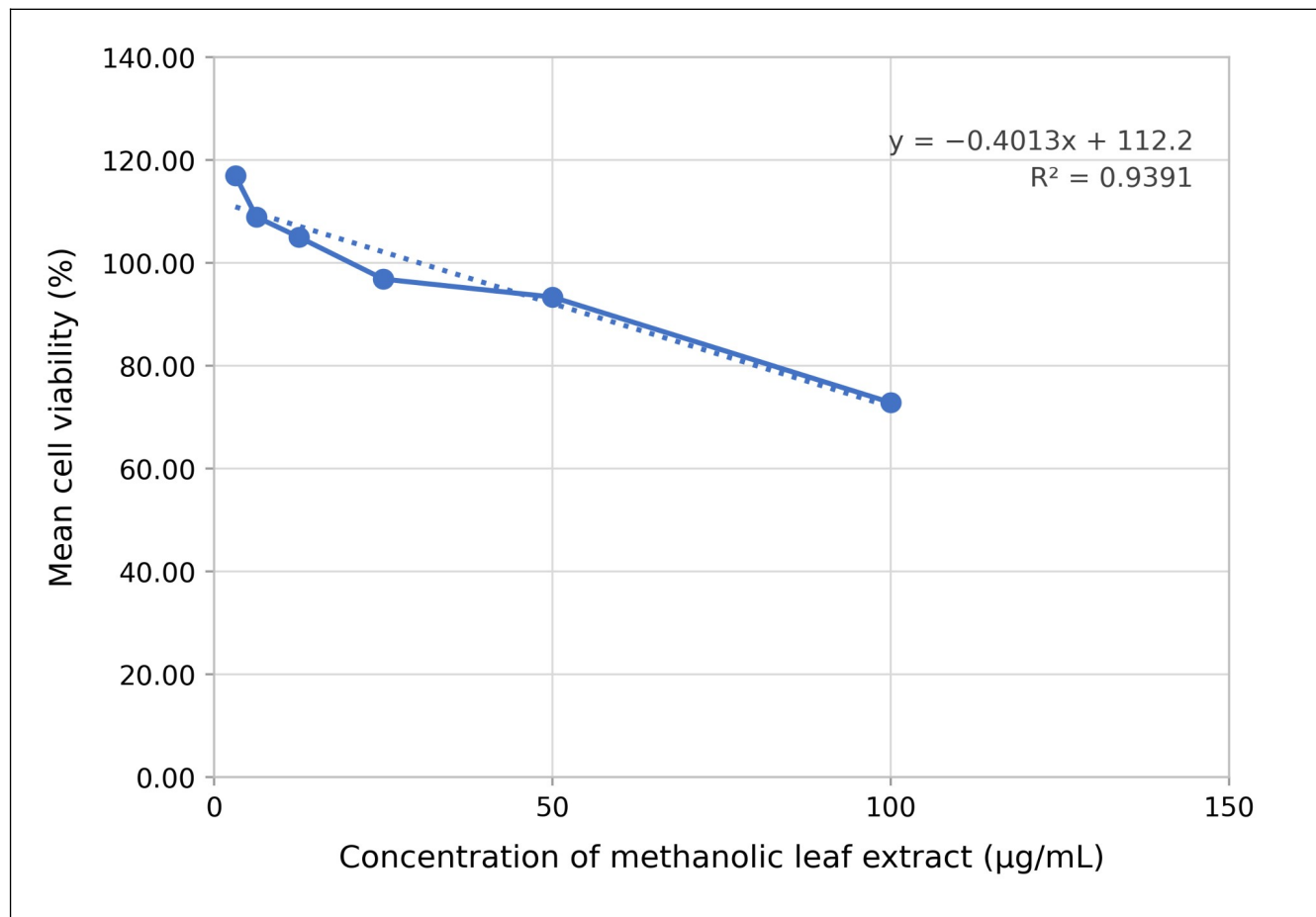


Fig. 2. Concentration–response relationship for the methanolic leaf extract of *Simarouba glauca* (MSG-01) against L929 mouse fibroblasts after 24 hr exposure (MTT assay). Points are the mean of three independent experiments, expressed as a percentage of the untreated control; the corresponding standard deviations are given in Table 4. The dotted line is the least-squares linear fit to the measured data ($y = -0.4013x + 112.2$; $R^2 = 0.9391$) and is drawn only across the concentration range actually tested; it has deliberately not been extended beyond 100 µg/mL. Viability remained above the 70 % threshold below which a material is classified as cytotoxic under ISO 10993-5 at every concentration examined. The IC_{50} of 155.1 µg/mL quoted in the text is the concentration at which this fitted line reaches 50 % viability; that value lies outside the range tested and is therefore an extrapolated estimate and for that reason it is not plotted.

Tannins act largely through non-specific binding. Their multiple phenolic hydroxyls form hydrogen bonds and hydrophobic contacts with bacterial proteins, precipitating extracellular enzymes and inactivating cell-surface adhesins and they chelate the iron required for bacterial respiration and ribonucleotide reduction. Because this mechanism does not depend on a single molecular target, resistance emerges slowly, but the same non-specificity limits selectivity. Simple phenolics partition into the lipid bilayer and increase membrane permeability, causing leakage of potassium ions and intracellular metabolites and at higher concentrations denature cytoplasmic proteins (6). Alkaloids intercalate into bacterial DNA and inhibit topoisomerases. Terpenoids, being lipophilic,

accumulate in the membrane and disorder acyl-chain packing, increasing fluidity and permeability (8).

In the present study terpenoids were the only class detected in all four extracts, including hexane, yet the hexane extract was entirely inactive. This is informative rather than contradictory: it indicates that the terpenoid fraction alone, at the amounts delivered here, was insufficient to inhibit growth and that activity depended on the polar constituents that hexane failed to recover. The activity therefore tracked the recovery of polar constituents across the solvent series, which is consistent with these classes contributing to the effect, although the screen cannot attribute activity to any individual constituent.

The trend towards greater susceptibility among the Gram-positive strains is explicable on structural grounds, even though it did not reach significance with only two organisms per group. The Gram-positive envelope presents a thick but porous peptidoglycan layer that does not exclude moderately sized polar molecules, whereas the Gram-negative outer membrane presents an asymmetric bilayer whose outer leaflet is composed of lipopolysaccharide. This layer restricts the entry of hydrophobic compounds, while hydrophilic solutes must traverse porin channels that exclude molecules above approximately 600 Da. Gram-negative organisms additionally express resistance-nodulation-division efflux systems, notably AcrAB–TolC in *E. coli* and MexAB–OprM in *P. aeruginosa*, which extrude plant phenolics and flavonoids before they reach an inhibitory intracellular concentration (4). The comparatively poor susceptibility of *P. aeruginosa*, the least susceptible of the four organisms at every amount tested, is consistent with its unusually low outer-membrane permeability and constitutive efflux capacity.

These observations agree with reports that aqueous extracts rich in phenolic compounds disrupt bacterial membranes and increase permeability and that flavonoid-rich extracts demonstrate broad-spectrum activity, while phenolics and flavonoids form complexes with bacterial proteins, disrupting enzyme activity and interfering with microbial metabolism (6, 26, 27).

The non-monotonic dose response

The largest zones were consistently recorded at 75 μL rather than at 100 μL and the chloroform extract was active only at the two lowest amounts; the significant solvent $\times$ volume interaction confirms that this pattern was not uniform across extracts. Because all wells received the same 10 mg/mL stock, the effect cannot be attributed to concentration-dependent diffusion: the concentration of extract entering the agar was identical in every well and only the total amount delivered differed. A more plausible explanation is overfilling. A well of 6 mm diameter and 4 mm depth has a capacity of approximately 113 μL , so a 100 μL load fills it close to the rim and surface spillage during handling would disperse extract as a lateral film instead of allowing radial diffusion from a defined reservoir, producing smaller and less regular measured zones. Since a genuine loss of antibacterial activity with increasing dose has no ready biological explanation, we regard this as a methodological artefact rather than a pharmacological effect. It is acknowledged as a limitation and MIC determination by broth microdilution—which is independent of diffusion and fill volume—is identified below as the appropriate next step.

Cytotoxicity and biocompatibility

The methanolic leaf extract showed low toxicity towards L929 fibroblasts, viability remaining above 70 % at the highest concentration tested (Table 4, Fig. 2), which classifies it as non-cytotoxic under ISO 10993-5 (24). The extrapolated IC_{50} of 155.1 $\mu\text{g/mL}$ lies above the highest concentration tested and cannot by itself

establish a safety margin. Its value here is comparative rather than absolute: it indicates that any cytotoxic threshold lies well above the concentrations at which antibacterial activity was recorded. Confirmation would require the assay to be repeated over an extended range, which we identify as a priority for further work.

One feature of the assay design should be stated explicitly. The extract was delivered in DMSO at a final concentration of 2.5 % (v/v) and no vehicle-only control was included. Dimethyl sulfoxide at this concentration is itself mildly cytotoxic to L929 fibroblasts, so the viability values reported here reflect the combined effect of extract and vehicle, measured against a control containing neither. Because the DMSO concentration was held constant across the entire concentration series, this contribution was identical in every treated well and cannot account for the concentration-dependent trend; but it does mean that the values in Table 4 systematically understate the viability attributable to the extract alone. The consequence is conservative: the true cytotoxicity of the extract is lower than reported and the classification as non-cytotoxic under ISO 10993-5 is therefore reached with a margin rather than marginally. A vehicle control will be included in subsequent work to quantify this offset.

Viability exceeded that of the untreated control at concentrations of 12.5 $\mu\text{g/mL}$ and below, rising to 117.00 ± 1.15 % at 3.125 $\mu\text{g/mL}$, a statistically significant increase. The trend was consistent across the three independent experiments and the associated standard deviations were small, which argues against random variation; and since the constant DMSO burden would if anything depress these values, the underlying stimulation may be greater than measured. Two explanations are commonly advanced. The first is genuine hormesis: low concentrations of plant phenolics and flavonoids can stimulate proliferation or activate cytoprotective signalling, whereas the same compounds are inhibitory at higher concentrations, giving the biphasic response characteristic of polyphenol-rich extracts (28). The second is an assay artefact, since the MTT readout reports mitochondrial dehydrogenase activity rather than cell number and any compound that raises metabolic rate or that is itself capable of reducing tetrazolium salts—will inflate apparent viability. Phenolic compounds and reducing sugars, both detected in the methanolic extract in the present screen (Table 1), are known to reduce MTT directly. The present data cannot distinguish between these possibilities; doing so would require a cell-free control in which extract and MTT reagent are incubated without cells, together with an orthogonal endpoint such as neutral red uptake or a direct DNA-based cell count. Until such controls are available, we regard the apparent stimulation as unresolved and do not interpret it as evidence of a proliferative effect.

Comparison with earlier work on *Simarouba glauca*

Several groups have reported antibacterial activity for *S. glauca* leaf extracts and the present findings should



be read against them (Table 5). Extracts were screened phytochemically and reported to exhibit antimicrobial activity but did not compare solvents systematically or assess mammalian cytotoxicity (11). Phytochemical composition and antimicrobial properties were examined and, as here, the polar fractions were found

to be richer in phenolics and flavonoids (12). Broad-spectrum antimicrobial, antioxidant and anticancer activity was reported (13). Cytotoxic, antibacterial, antioxidant, anthelmintic and anticoagulant activities were reported for leaf phytochemicals (14).

Table 5. Comparison of the present study with previously published investigations of *Simarouba glauca* leaf extracts

Study	Solvents compared	Test organisms	Cytotoxicity assessed	Principal finding
Kumar et al. (11), 2012	Single extract series	Bacterial and fungal panel	No	Phytochemical constituents and antimicrobial activity reported
Kumari et al. (12), 2019	Polar and non-polar extracts	Bacterial panel	No	Polar extracts richer in phenolics and flavonoids
Ramasamy et al. (13), 2022	Ethanol and methanol	14 bacterial, 6 fungal strains	Yes (anticancer)	Methanolic extract most active; MIC 3.2 mg/mL; GC–MS and FTIR profiling; synergy with antibiotics tested
Alshehri et al. (14), 2024	Leaf phytochemical fraction	Bacterial panel	Yes	Cytotoxic, antibacterial, antioxidant, anthelmintic and anticoagulant activity
Present study	Aqueous, methanol, chloroform, hexane	<i>S. aureus</i> , <i>S. mutans</i> , <i>E. coli</i> , <i>P. aeruginosa</i>	Yes (L929, ISO 10993-5)	Aqueous > methanolic > chloroform > hexane; methanolic extract non-cytotoxic

Two points of divergence warrant further discussion. First, the findings indicate that the aqueous extract exhibited greater activity than the methanolic extract against all four organisms. This observation differs from the general trend reported in the literature, where alcoholic extracts are typically found to demonstrate greater antimicrobial activity. It is worth noting that the methanolic extract gave the higher extraction yield (33.3 % against 24.0 % w/w), so its lower activity is not attributable to a smaller mass of recovered material; the aqueous solvent appears to have recovered a more active fraction rather than simply more of it. Geographical and seasonal variation in metabolite content is a plausible explanation: the material used here was collected at a single site in Belagavi in December, in a tropical climate receiving approximately 1468 mm of annual rainfall and phenolic accumulation in Simaroubaceae varies with soil, altitude and season. Differences in extraction protocol are a second contributor. It was not possible to distinguish between these explanations without quantitative compositional data and we present the observation as one requiring confirmation across collection sites and seasons.

The inclusion of a full streptomycin dilution series allows the extract activity to be placed on a common scale: the aqueous extract achieved roughly half to three-quarters of the zone produced by

undiluted streptomycin, depending on the organism, which is a substantial effect for a crude, unfractionated extract but does not approach the potency of a purified aminoglycoside. Second, the zones of inhibition reported here are of the same order as those in earlier reports, but direct numerical comparison is not legitimate, because the amount of extract delivered per well, the well dimensions and the inoculum density differ between studies and few reports state all three. This is a general weakness of the agar diffusion literature and a further argument for MIC-based comparison.

The antibacterial activity in *S. glauca* leaf extracts and cytotoxicity were documented previously (13, 14). By preparing four extracts from a single batch of leaf material and testing them against a common organism panel under identical conditions, our findings establish a polarity–activity relationship for this species that could not be elucidated through single-solvent studies. Analysis of variance across the full experimental design further supported this relationship and identified the aqueous extract, rather than the methanolic extract, as exhibiting the greatest activity. By assessing biocompatibility against ISO 10993-5 rather than reporting an IC₅₀ alone, we place the cytotoxicity result in the regulatory framework used for materials intended for tissue contact. And by including *Streptococcus mutans* in the panel we

identify a specific application for which the observed profile is directly relevant: activity against the principal cariogenic organism combined with non-cytotoxicity towards fibroblasts is the profile sought in candidate constituents for oral-care formulations, a direction not previously pursued for this species.

Limitations of the study

Four limitations qualify these findings. First, the phytochemical characterisation was qualitative. Colour and precipitation reactions establish which classes of metabolite are present above the detection threshold of a particular reagent, but they are not specific to single compounds, several reagents respond to more than one class and they give no measure of abundance. The screen can therefore show that the polar extracts recovered a broader range of metabolite classes and that this parallels the antibacterial ranking, but it cannot establish which constituents are responsible or in what proportion. Causal language has accordingly been avoided throughout. Establishing causation requires bioassay-guided fractionation with activity tracked through each separation step, quantification of total phenolic and flavonoid content and identification of individual constituents by high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC–MS).

Second, the agar well diffusion assay is a screening method. It ranks extracts but does not yield a MIC and its readout depends on diffusion characteristics as well as intrinsic potency, so poorly diffusing or viscous extracts may be underestimated. Broth microdilution to obtain MIC and minimum bactericidal concentration (MBC) values is required before any comparison with reference antibiotics is meaningful and the absence of such values here is why we do not attempt one.

Third, the cytotoxicity assessment used a single cell line, a single exposure time, a concentration range that did not reach the IC_{50} and a vehicle concentration for which no separate control was run. A fuller safety assessment would require a second cell line, a longer exposure, a vehicle control, an orthogonal viability endpoint and confirmation that the extract does not reduce tetrazolium salts directly.

Fourth, the comparison between Gram-positive and Gram-negative susceptibility rests on two organisms in each group, which is insufficient for statistical inference; a larger panel would be required to test whether the trend observed here generalises. A related point concerns the solvent controls: methanol was run as the negative control against all four organisms and was inactive, but matched controls for water, chloroform and hexane were not run separately. Water is inert in this assay and the hexane extract produced no inhibition at any amount, so neither is a plausible source of artefact; the absence of a chloroform control is nonetheless a gap and the weak activity recorded for the chloroform

extract at the two lower amounts should be read with that in mind.

These limitations reflect the scope of the study, which was designed as a comparative preliminary screen to identify which solvent system merits detailed investigation. That question has been answered: the aqueous and methanolic extracts are the productive ones. The quantitative and mechanistic work identified above is in progress in this laboratory and will be reported separately.

5. Conclusion

Aqueous and methanolic Soxhlet extracts of *Simarouba glauca* leaves were richer in alkaloids, flavonoids, tannins, phenolic compounds, glycosides and terpenoids than chloroform and hexane extracts and this difference was reflected directly in their antibacterial performance. Analysis of variance confirmed a significant effect of extraction solvent for every organism tested ($p < 0.001$), with all four solvents separated from one another by Tukey's test. The aqueous extract was the most active against all four test organisms, giving its largest zone of inhibition against *Staphylococcus aureus*, while the hexane extract was inactive throughout. The methanolic extract did not reduce L929 fibroblast viability below 70 % at 100 $\mu\text{g/mL}$ and is therefore non-cytotoxic by ISO 10993-5 criteria.

Recommendation: The aqueous and methanolic leaf extracts of *S. glauca* merit progression to bioassay-guided fractionation with MIC and MBC endpoints, coupled with quantification of total phenolic and flavonoid content and chromatographic identification of the active constituents. Given the activity recorded against *Streptococcus mutans* alongside fibroblast biocompatibility under ISO 10993-5, evaluation of the aqueous extract in an oral-care formulation is a specific and testable application. Confirmation of the extrapolated IC_{50} over an extended concentration range, with a vehicle control included, should precede any *in vivo* work.

Acknowledgements

The authors are grateful to the Director, ICMR–National Institute of Traditional Medicine, Belagavi and to the Department of Health Research, Ministry of Health and Family Welfare, Government of India, for supporting the study and providing facilities.

Authors' contributions

PSK carried out the investigation, performed the experiments, conducted the formal analysis, interpreted the results and drafted the manuscript. HVH conceived the study, supervised the research, designed the study and critically reviewed the manuscript. MML participated in the study design,



developed the methodology, interpreted the data and critically revised the manuscript. SIJ contributed to the methodology, data curation, validation of experimental findings, interpretation of results and manuscript review. AC assisted with laboratory investigations, data curation, validation of experimental procedures and manuscript editing. AJ assisted with experimental work, data collection, data curation, quality control of laboratory procedures and manuscript review. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

Authors do not have any conflict of interests to declare.

Ethical issues

None

Declaration on the use of artificial intelligence and AI-assisted technologies

During the preparation of this manuscript the authors used QuillBot (QuillBot Inc.) solely to improve the grammar, readability and language of the text. No artificial intelligence tool was used to design the experiments, to generate, analyse or interpret data, to produce tables or figures, to conduct the literature search or to draft the scientific content of the manuscript. All data reported here were generated experimentally by the authors. After using this tool the authors reviewed, verified and edited the entire manuscript and take full responsibility for the content of the published article.

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