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Phytochemical Profiling, Antioxidant Potential, and Antimicrobial Properties of Medicinal Plants: A Comparative Study

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Abstract

Medicinal plants represent chemically diverse reservoirs of secondary metabolites with potential applications in pharmaceutical discovery. However, the translation of plant-derived bioactivity into scientifically defensible therapeutic leads remains constrained by inconsistent extraction procedures, incomplete chemical characterisation, assay-dependent biological measurements and inadequate assessment of cytotoxicity. These limitations are particularly important in research addressing antimicrobial resistance (AMR) and oxidative stress, where preliminary activity is frequently interpreted without sufficient consideration of chemical identity, selectivity or biological relevance. This study develops an integrated comparative framework for investigating the phytochemical composition, antioxidant capacity, antimicrobial activity and preliminary safety profiles of five medicinal plants: *Azadirachta indica*, *Ocimum tenuiflorum*, *Curcuma longa*, *Moringa oleifera* and *Camellia sinensis*. The proposed experimental design combines authenticated botanical material, standardised multi-solvent extraction, quantitative total phenolic and flavonoid determination, untargeted liquid chromatography–tandem mass spectrometry (LC-MS/MS), feature-based molecular networking, complementary antioxidant assays and antimicrobial susceptibility testing. Bacterial activity is evaluated through broth microdilution, while yeast susceptibility is assessed using an appropriate antifungal reference methodology. Cytotoxicity testing provides a preliminary measure of extract selectivity. Multivariate statistical analysis and a proposed Integrated Phytochemical–Bioactivity–Selectivity Framework (IPBSF) are introduced to connect chemical fingerprints with biological performance. The proposed framework moves beyond conventional extract ranking by distinguishing chemical abundance, antioxidant performance, antimicrobial potency and mammalian-cell selectivity. It also introduces a structured strategy for identifying metabolite families associated with biological activity, evaluating the stability of candidate rankings and prioritising extracts for subsequent bioassay-guided fractionation. The framework provides a reproducible approach to comparative medicinal-

plant research. Its principal contribution is methodological and analytical rather than a claim of demonstrated therapeutic efficacy. Experimental validation is necessary before any plant or extract can be identified as a superior antimicrobial or antioxidant candidate.

Keywords: Medicinal Plants; Phytochemical Profiling; LC-MS/MS; Metabolomics; Antioxidant Capacity; Antimicrobial Resistance; Molecular Networking; Cytotoxicity; Bioactivity-Guided Fractionation; Natural-Product Discovery

Background and Scientific Context

Natural products have maintained an important position in pharmaceutical research because of their structural diversity, biological complexity and capacity to provide molecular scaffolds for therapeutic development. Newman and Cragg (2020) demonstrate the continuing contribution of natural products and natural-product-derived compounds to modern drug discovery. More recently, Atanasov et al. (2021) emphasised that technological advances in analytical chemistry, molecular biology, metabolomics and computational screening have created new opportunities for investigating natural substances as sources of pharmacologically relevant molecules.

Plants are particularly important within this research landscape. Their secondary metabolites include phenolic acids, flavonoids, terpenoids, alkaloids, tannins, glycosides and other chemically diverse compounds. These substances frequently perform ecological functions associated with plant defence, environmental adaptation and interactions with microorganisms. Their structural characteristics may also generate biological activities relevant to human health, including radical scavenging, enzyme inhibition, membrane interaction and modulation of microbial growth (Dias et al., 2021; Ullah et al., 2020).

Nevertheless, the identification of biological activity in a crude plant extract should not be confused with the identification of a therapeutically useful compound. A positive antioxidant assay does not establish protection against oxidative damage in human tissues, while antimicrobial inhibition under laboratory conditions does not demonstrate clinical effectiveness. The transition from preliminary screening to pharmaceutical development requires chemical identification, reproducibility, mechanistic investigation, safety assessment and evidence of biological availability.

This distinction establishes the central scientific problem addressed by the present study: medicinal-plant research requires an integrated analytical approach capable of connecting the chemical composition of extracts with their measured biological effects and preliminary safety characteristics.

Antimicrobial Resistance and the Importance of Plant-Derived Compounds

Antimicrobial resistance represents an increasingly serious challenge for healthcare systems. The Global Burden of Disease 2021 Antimicrobial Resistance Collaborators (2024) estimated that bacterial antimicrobial resistance was associated with approximately 4.71 million deaths in 2021, including approximately 1.14 million deaths directly attributable to resistance. These estimates reinforce the need to investigate alternative approaches to antimicrobial discovery, particularly against organisms associated with difficult-to-treat infections.

Plant-derived compounds are of interest because their antimicrobial activities may involve different molecular targets and physiological processes. Álvarez-Martínez et al. (2021) describe several mechanisms through which plant compounds and extracts may influence bacterial activity, including alterations in membrane integrity, interference with cellular processes and modulation of mechanisms associated with microbial survival.

Phenolic compounds and flavonoids have received particular attention because their chemical structures may facilitate interactions with microbial membranes, proteins and other cellular components. However, their biological effects depend on molecular structure, concentration, solubility, microbial species and experimental conditions. Consequently, the antimicrobial properties of a plant extract cannot be reliably predicted from the total quantity of phenolic compounds alone.

A further challenge concerns differences between Gram-positive and Gram-negative bacteria. The outer membrane of Gram-negative organisms can restrict the penetration of numerous antimicrobial substances, whereas Gram-positive bacteria lack this additional permeability barrier. Although this distinction provides a reasonable hypothesis for comparative screening, it should not be treated as a universal rule because individual microorganisms differ in their resistance mechanisms, membrane composition, efflux systems and physiological characteristics.

The present research therefore proposes a comparative assessment of representative Gram-positive and Gram-negative organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), alongside selected fungal organisms. The objective is not simply to identify inhibition but to determine whether particular chemical profiles are consistently associated with antimicrobial potency.

Oxidative Stress and the Assessment of Antioxidant Potential

Reactive oxygen species are involved in physiological signalling and cellular regulation. However, excessive or inadequately controlled production of reactive oxygen species can contribute to oxidative damage affecting lipids, proteins and nucleic acids. Sies and Jones (2020) explain that reactive oxygen species have context-dependent biological functions and should not be understood exclusively as harmful substances.

Plant-derived polyphenols are frequently investigated because of their capacity to participate in redox reactions, donate electrons or hydrogen atoms, chelate certain metal ions and influence endogenous antioxidant defence pathways. Rudrapal et al. (2022) discuss the potential protective activities of dietary polyphenols and their relevance to oxidative-stress-associated conditions.

Despite their widespread use, chemical antioxidant assays provide only limited information about physiological antioxidant effectiveness. DPPH, ABTS and FRAP measure different aspects of chemical reducing or radical-scavenging behaviour. Their results are influenced by solvent systems, reaction conditions, molecular structure and reaction kinetics (Gulcin, 2020; Munteanu & Apetrei, 2021).

This methodological distinction is particularly important when comparing chemically different medicinal plants. A catechin-rich extract may perform strongly in certain radical-scavenging assays, whereas another extract containing compounds with different redox properties may demonstrate a distinct FRAP response. Such differences should be interpreted as complementary chemical characteristics rather than inconsistencies.

Accordingly, the proposed study uses multiple antioxidant assays to generate a broader assessment of in vitro chemical antioxidant capacity. Where resources permit, a cellular oxidative-stress model should be introduced in a subsequent validation phase to investigate whether chemical antioxidant performance translates into measurable cellular protection.

Limitations of Conventional Comparative Phytochemical Studies

Despite substantial research on medicinal plants, several methodological weaknesses continue to restrict the comparability and translational interpretation of published findings.

First, extraction procedures vary considerably. Differences in solvent polarity, extraction duration, temperature, plant material, particle size and solid-to-solvent ratio influence the recovery of

secondary metabolites. Consequently, two studies investigating the same species may produce chemically different extracts even when they use identical plant parts.

Second, many comparative studies depend heavily on qualitative phytochemical screening and total phenolic or flavonoid measurements. Although these methods are useful for preliminary characterisation, they cannot identify the specific molecular constituents responsible for biological activity. The Folin–Ciocalteu assay, for example, measures overall reducing capacity under its reaction conditions and is not exclusively specific to phenolic substances.

Third, antioxidant performance is sometimes represented through a single assay. Such an approach overlooks the mechanistic differences between antioxidant measurement systems and may generate misleading comparisons.

Fourth, antimicrobial screening frequently relies on agar diffusion without sufficiently establishing minimum inhibitory concentrations. Diffusion depends on molecular size, polarity, solubility and movement through agar; therefore, inhibition-zone diameter is not a direct measure of antimicrobial potency across chemically different extracts.

Fifth, biological activity is frequently reported without adequate assessment of cytotoxicity. An extract capable of inhibiting bacterial growth may also damage mammalian cells. Without a selectivity assessment, the pharmaceutical relevance of an apparently active extract remains uncertain.

Finally, chemical profiling and biological testing are often presented as separate investigations. This separation makes it difficult to determine whether particular molecular families contribute meaningfully to observed biological effects.

These limitations provide the methodological justification for the integrated framework developed in this article.

Research Gap

The principal research gap is not the absence of studies reporting antioxidant or antimicrobial activity in the five selected plants. Each species has an established literature concerning its phytochemical composition and traditional or experimental biological properties.

Rather, the gap concerns the insufficient integration of four analytical dimensions within a harmonised comparative design:

1. Standardised extraction and botanical characterisation.
2. Molecular-level chemical profiling and metabolite annotation.
3. Complementary antioxidant and antimicrobial measurements.
4. Mammalian-cell selectivity and quantitative chemistry–activity relationships.

Existing chemical characterisation recommendations, including the ConPhyMP guidelines developed by Heinrich et al. (2022), provide an important foundation for improving the reproducibility of medicinal-plant research. Similarly, feature-based molecular networking offers opportunities to investigate relationships among related chemical features rather than treating every detected mass-spectrometric signal as an isolated observation (Nothias et al., 2020).

The present study brings these analytical approaches together within a comparative prioritisation framework. Its contribution lies in the proposed integration and validation strategy, not in claiming that metabolomics, antioxidant assays or antimicrobial testing are individually new.

Research Objectives

The principal objective is to establish an integrated comparative framework for evaluating the phytochemical composition and biological performance of five medicinal plants.

The specific objectives are:

1. To investigate the influence of extraction-solvent polarity on the chemical composition and extraction yield of the selected medicinal plants.
2. To quantify total phenolic and flavonoid contents and compare their relationships with antioxidant measurements.
3. To characterise major chemical features using untargeted LC-MS/MS and feature-based molecular networking.
4. To evaluate antioxidant capacity using DPPH, ABTS and FRAP assays under standardised experimental conditions.
5. To determine antimicrobial activity against selected Gram-positive, Gram-negative and fungal reference organisms.
6. To investigate preliminary mammalian-cell cytotoxicity and calculate organism-specific selectivity indices.
7. To identify chemical features and metabolite families associated with measured antioxidant and antimicrobial outcomes.
8. To develop and evaluate an integrated prioritisation framework incorporating chemical evidence, biological activity and preliminary safety.

Research Questions

RQ1: How do plant species and extraction-solvent polarity influence the phytochemical composition and biological performance of the selected medicinal plants?
RQ2: To what extent are individual metabolite features and molecular-network clusters associated with antioxidant capacity, antimicrobial potency and mammalian-cell selectivity?
RQ3: Can an integrated chemical–bioactivity–selectivity framework provide a more reliable basis for prioritising medicinal-plant extracts than conventional single-assay rankings?

Research Hypotheses

H1: Extraction-solvent polarity produces significant differences in the phytochemical composition and measured biological activity of the five medicinal plants.
H2: Total phenolic and flavonoid contents are positively associated with antioxidant performance, although the strength of the relationship differs among antioxidant assays.
H3: Specific metabolite features and molecular-network clusters explain variation in antimicrobial potency more effectively than bulk total phenolic measurements alone.
H4: Gram-positive bacterial strains demonstrate greater susceptibility to selected plant extracts than Gram-negative strains, although the magnitude of this difference varies according to extract composition and microbial species.
H5: Incorporating mammalian-cell selectivity into comparative prioritisation changes the ranking of extracts relative to rankings based exclusively on antioxidant or antimicrobial potency.
H6: Intermediate-polarity extraction fractions demonstrate favourable integrated biological performance; however, the direction and magnitude of this relationship are species-dependent and must be established experimentally.

Comparative Phytochemical Basis of the Selected Medicinal Plants

Rationale for Plant Selection

The selection of *Azadirachta indica*, *Ocimum tenuiflorum*, *Curcuma longa*, *Moringa oleifera* and *Camellia sinensis* is based on three considerations: their established importance in traditional medicinal systems, their chemically differentiated metabolite profiles and their suitability for comparative extraction and biological investigation.

The five species provide a useful comparative model because they represent different plant families and contain distinct classes of secondary metabolites. Their selection permits investigation of whether chemical diversity corresponds to differences in antioxidant performance, antimicrobial activity and preliminary safety.

Importantly, the selection does not imply that all five plants possess equivalent pharmacological potential or that their traditional medicinal uses establish clinical efficacy. Their biological characteristics must be evaluated through reproducible experimentation.

Azadirachta Indica: Limonoid-Rich Phytochemical Diversity

Azadirachta indica, commonly known as neem, belongs to the family Meliaceae. Its leaves contain chemically diverse constituents, including limonoids, flavonoids and phenolic compounds. Limonoids are particularly relevant to investigations of plant defence and biological activity because of their structurally complex terpenoid-derived chemical architecture.

The potential antimicrobial activity of neem-derived extracts may involve several metabolite classes rather than a single constituent. Consequently, a comparative investigation should avoid attributing the biological effects of a crude extract exclusively to azadirachtin or another well-known neem compound without direct chemical evidence.

The present framework proposes investigating whether limonoid-associated molecular features correlate with antimicrobial outcomes after controlling for extract concentration, solvent effects and overall chemical composition.

Ocimum Tenuiflorum: Phenylpropanoids and Phenolic Constituents

Ocimum tenuiflorum, also known as holy basil or tulsi, belongs to the family Lamiaceae. Its phytochemical composition includes phenylpropanoids, phenolic acids and terpenoid constituents. Eugenol and rosmarinic acid are among the compounds commonly associated with its chemical profile.

The coexistence of phenolic and volatile constituents makes this plant particularly relevant to comparative extraction research. Different extraction solvents may recover substantially different proportions of these chemical families.

The research should therefore distinguish the chemical characteristics of the extracts rather than assuming that a single solvent system provides a complete representation of the plant's phytochemical composition.

Curcuma Longa: Curcuminoids and Sesquiterpenes

Curcuma longa, belonging to the family Zingiberaceae, is widely investigated for its rhizome-derived curcuminoids and volatile sesquiterpenes. Curcumin is particularly prominent in pharmaceutical and natural-product research because of its chemical and biological properties.

However, curcumin presents important translational challenges associated with limited aqueous solubility, chemical stability and bioavailability. Stohs et al. (2020) discuss formulation strategies intended to improve the availability of curcumin-based preparations.

These limitations demonstrate why in vitro antioxidant or antimicrobial activity should not be interpreted as evidence of therapeutic suitability. The proposed study will distinguish the chemical abundance and laboratory activity of curcuminoid-containing extracts from their potential pharmaceutical applicability.

Moringa Oleifera: Flavonoids and Glucosinolate-Derived Constituents

Moringa oleifera, a member of the family Moringaceae, contains several classes of phytochemicals, including flavonoid glycosides, phenolic acids and glucosinolate-derived compounds.

Pareek et al. (2023) provide a comprehensive account of its phytochemistry, pharmacological investigation and toxicological considerations. Its leaf extracts are particularly relevant to comparative antioxidant research because of their reported polyphenolic constituents.

A further analytical consideration concerns the extraction and stability of glucosinolate-derived compounds. Their recovery may depend on plant processing, enzymatic activity and extraction conditions. Therefore, the experimental design should record processing conditions and avoid treating the chemical composition of all moringa extracts as interchangeable.

Camellia Sinensis: Catechins and Flavonoid Chemistry

Camellia sinensis, belonging to the family Theaceae, is an important source of catechins, including epigallocatechin gallate (EGCG), as well as other flavonoids and methylxanthines.

Its well-characterised polyphenolic composition makes it a valuable comparative reference within the selected plant panel. However, the designation of green tea as a strong antioxidant reference should not be interpreted as a predetermined experimental result.

Differences in processing, leaf quality, extraction conditions and catechin stability may substantially influence the measured activity of individual preparations.

Comparative Analytical Rationale

The principal advantage of comparing these five plants is the opportunity to investigate whether different phytochemical architectures produce distinct patterns of biological performance.

Plant	**Principal metabolite families to investigate**	**Main analytical interest**
Azadirachta indica	Limonoids, flavonoids, phenolic acids	Relationship between limonoid-associated features and antimicrobial activity
Ocimum tenuiflorum	Phenylpropanoids, rosmarinic acid, terpenoids	Relationship between phenolic composition and antioxidant and antimicrobial outcomes
Curcuma longa	Curcuminoids, sesquiterpenes	Solvent-dependent chemical recovery and bioactivity
Moringa oleifera	Flavonol glycosides, phenolic acids, glucosinolate derivatives	Chemical diversity and antioxidant performance
Camellia sinensis	Catechins, flavonols, methylxanthines	Catechin-associated antioxidant activity and comparative chemical profiling

The proposed comparison is therefore designed around chemical differentiation rather than the assumption that one plant will necessarily outperform all others.

Materials and Methods

Research Design and Analytical Strategy

The study is designed as a comparative laboratory investigation employing a factorial experimental structure. The principal experimental factors are plant species and extraction-solvent system, while the measured outcomes include extraction yield, phytochemical composition, antioxidant performance, antimicrobial potency and mammalian-cell cytotoxicity.

The five selected plants constitute the botanical factor, while three solvent systems represent different chemical-polarity ranges. The resulting experimental design contains 15 primary plant–solvent combinations.

Three independently prepared biological extraction replicates should be used as an initial design, with technical replicates for individual analytical measurements. However, the final sample size must be established through an appropriate power analysis, particularly for the high-dimensional metabolomics component. Independent collections, extraction batches and technical measurements must be distinguished clearly.

The experimental workflow comprises six interconnected stages:

1. Botanical authentication and preparation of plant material.
2. Standardised extraction and determination of extraction yield.
3. Preliminary and quantitative phytochemical analysis.
4. Untargeted LC-MS/MS profiling and molecular-network analysis.
5. Antioxidant, antimicrobial and cytotoxicity evaluation.
6. Statistical integration and extract prioritisation.

The analytical sequence is intended to minimise methodological variation and permit meaningful comparisons across plant species and extraction systems.

Plant Material Collection and Botanical Authentication

Leaves of Azadirachta indica, Ocimum tenuiflorum, Moringa oleifera and Camellia sinensis, together with rhizomes of Curcuma longa, should be obtained from documented collection locations.

Botanical identification must be performed by a qualified taxonomist. Voucher specimens should be deposited in a recognised herbarium, and the accession numbers should be included in the final publication.

The collection record should contain:

- Accepted botanical name and family.
- Plant part used.
- Collection location and date.
- Geographical coordinates where permitted.
- Collection season and phenological stage.
- Authentication method and responsible taxonomist.
- Voucher specimen number.
- Relevant collection permissions and access-and-benefit-sharing compliance.

Where feasible, DNA barcoding should supplement morphological identification, particularly where closely related species or commercial plant materials create uncertainty.

Collected materials should be cleaned using a documented procedure, dried under controlled conditions and ground to a consistent particle size. Drying temperature and duration must be reported because excessive heat can alter sensitive phytochemicals.

The processed material should be stored in airtight, light-protected containers under controlled conditions until extraction.

Standardised Extraction Procedures

Extraction represents a critical determinant of the chemical composition and biological activity of medicinal-plant preparations. The extraction procedure must therefore be standardised across all five species.

Three solvent systems are proposed:

1. Non-polar fraction: n-hexane.

2. Intermediate-polarity fraction: ethyl acetate.
3. Polar fraction: distilled water.

These solvents are selected to provide chemically differentiated extraction environments. Ethyl acetate is used as a defined intermediate-polarity solvent rather than alternating between ethyl acetate and aqueous ethanol, as occurred in the original manuscript.

Ultrasound-assisted extraction is proposed because it can improve solvent penetration and mass transfer under controlled conditions. The extraction parameters should be established through preliminary optimisation and then held constant across the experimental groups.

A consistent solid-to-solvent ratio should be used. Extraction duration, temperature, ultrasound frequency and power, filtration conditions and concentration procedures must be recorded.

After extraction, organic solvents should be removed using an appropriate reduced-pressure evaporation procedure. Aqueous extracts may be freeze-dried to obtain stable dry material.

Extraction yield should be calculated using the following equation:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dry extract}}{\text{Mass of dry plant material}} \times 100$$

The resulting extracts should be weighed, coded and stored under conditions that minimise degradation.

Each extract should be reconstituted using a solvent compatible with the subsequent analytical method. Dimethyl sulfoxide may be used where appropriate for biological assays, but its final concentration must be controlled and independently tested against each microorganism and cell model.

A major consideration is that different extracts may have different solubility characteristics. Extracts should not be treated as directly comparable if one is fully dissolved while another remains suspended or precipitates during testing.

Preliminary Phytochemical Screening

Qualitative phytochemical screening will provide an initial indication of the major metabolite classes present in the extracts.

The screening should include tests for:

- Alkaloids.
- Flavonoids.
- Phenolic compounds and tannins.
- Terpenoids and steroids.
- Saponins.
- Glycosides.

These procedures may follow established preliminary phytochemical methods described by Shaikh and Patil (2020).

However, qualitative reactions should be treated as screening evidence rather than definitive compound identification. Colour formation and precipitation reactions may be affected by interfering substances, and a positive qualitative test does not establish the presence or concentration of a specific molecule.

Consequently, the qualitative findings will be interpreted alongside chromatographic and mass-spectrometric characterisation.

Quantification of Total Phenolic Content

Total phenolic content will be determined using the Folin–Ciocalteu colorimetric procedure.

Gallic acid will serve as the reference standard, and results will be expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g).

A calibration curve should be prepared using multiple gallic acid concentrations within the validated analytical range.
All samples should be analysed using appropriate blanks, calibration controls and technical replicates.
An important methodological limitation concerns the non-specific reducing capacity measured by the Folin–Ciocalteu reagent. Certain non-phenolic reducing substances may contribute to the observed response. Accordingly, the results should be described as Folin–Ciocalteu-reactive total phenolic equivalents rather than absolute measurements of all phenolic molecules.

Quantification of Total Flavonoid Content
Total flavonoid content will be assessed using an aluminium chloride-based colorimetric method. Quercetin will be used as the reference standard, with results reported as milligrams of quercetin equivalents per gram of dry extract (mg QE/g).
Because flavonoid subclasses differ in their ability to form complexes with aluminium ions, the method does not provide a complete quantitative measurement of all flavonoids.
The results will therefore be interpreted as operational estimates and compared with LC-MS/MS findings to determine whether measured flavonoid equivalents reflect the identified molecular composition.

Untargeted LC-MS/MS Profiling
Untargeted liquid chromatography–tandem mass spectrometry will be used to investigate the molecular composition of the extracts.
The proposed analytical platform comprises ultra-high-performance liquid chromatography coupled with a high-resolution mass spectrometer, such as a Q-TOF or Orbitrap instrument.
Both positive and negative electrospray ionisation modes should be considered to improve the coverage of chemically diverse metabolites.
Chromatographic separation will be performed using a suitable reversed-phase column and validated mobile-phase conditions. The analytical method should be optimised according to the chemical characteristics of the five plant extracts.
Quality assurance procedures should include:

- Solvent blanks.
- Extraction blanks.
- Pooled quality-control samples.
- Internal standards where appropriate.
- Randomised injection order.
- Repeated QC injections throughout the analytical sequence.
- Monitoring of retention-time and mass accuracy stability.
- Evaluation of feature reproducibility and missingness.

Raw data will undergo peak detection, chromatographic alignment, isotope grouping, adduct annotation and background correction.
Feature abundance will be normalised using a documented procedure, with particular attention to batch effects and analytical drift.

Feature-Based Molecular Networking
Feature-based molecular networking will be performed through the Global Natural Products Social Molecular Networking platform.
Nothias et al. (2020) introduced feature-based molecular networking as an approach that combines chromatographic feature information with tandem mass-spectral similarity. This approach is

particularly useful for investigating related chemical families and resolving chromatographically distinct features.

The resulting network will contain nodes representing detected molecular features and edges representing spectral similarity.

The network will be examined according to:

1. Plant species.
2. Extraction-solvent system.
3. Relative feature abundance.
4. Antioxidant performance.
5. Antimicrobial activity.
6. Cytotoxicity and selectivity.

Reference-library matches will be evaluated critically. Mass-spectral similarity alone is insufficient to establish an unequivocal molecular identity.

Metabolite annotations will follow the confidence framework described by the Metabolomics Standards Initiative (Sumner et al., 2007). Authentic analytical standards should be used to confirm the identity of important candidate compounds where available.

The distinction between a confirmed compound, a probable annotation and an unidentified molecular feature must be preserved throughout the manuscript.

Antioxidant Activity Assessment

A complementary antioxidant assay panel will be employed to assess different aspects of chemical antioxidant performance.

DPPH Radical-Scavenging Assay

The DPPH assay will evaluate the capacity of the extracts to reduce the stable 2,2-diphenyl-1-picrylhydrazyl radical.

A series of extract concentrations will be tested to establish concentration-dependent responses.

Percentage radical-scavenging activity will be calculated using:

$$\text{Scavenging activity (\%)}= \frac{A_c-A_s}{A_c}\times100$$

Where:

- A_c represents the absorbance of the control.
- A_s represents the corrected absorbance of the sample.

Sample-specific colour blanks should be included to account for the natural pigmentation of the extracts.

The concentration required to produce 50% radical-scavenging activity will be estimated using an appropriate nonlinear concentration–response model.

The resulting IC_{50} values will be reported in micrograms per millilitre. Lower IC_{50} values indicate stronger activity under the specified assay conditions.

ABTS Radical-Cation Assay

The ABTS assay will measure the capacity of the extracts to reduce the ABTS radical cation.

The method offers a complementary analytical perspective because it accommodates a wider range of hydrophilic and lipophilic antioxidant compounds under suitable solvent conditions.

Trolox will be used as the reference antioxidant, and results will be expressed as Trolox-equivalent antioxidant capacity.

The assay conditions, reaction time, solvent composition and calibration range must be reported to support reproducibility.

Ferric Reducing Antioxidant Power

The FRAP assay will determine the capacity of the extracts to reduce ferric ions to ferrous ions under the specified acidic reaction conditions.

Results will be expressed as micromoles of ferrous-ion equivalents per gram of dry extract.

The FRAP assay provides information about electron-transfer reducing capacity but does not represent every possible antioxidant mechanism.

Integrated Interpretation of Antioxidant Outcomes

The three assays will be interpreted collectively.

DPPH, ABTS and FRAP should not be treated as interchangeable measurements. Differences between their outcomes may reveal variation in the reaction behaviour, polarity and chemical composition of the extracts.

Where resources permit, an additional oxygen radical absorbance capacity assay or a cell-based oxidative-stress experiment may provide further information.

The principal outcome of this stage will be a comparative antioxidant profile rather than a claim of demonstrated therapeutic antioxidant efficacy.

Antimicrobial Susceptibility Assessment

Antimicrobial activity will be assessed using reference microorganisms representing important bacterial and fungal groups.

The proposed bacterial panel includes:

- Staphylococcus aureus ATCC 29213.
- Methicillin-resistant Staphylococcus aureus ATCC 43300.
- Bacillus subtilis.
- Escherichia coli ATCC 25922.
- Pseudomonas aeruginosa ATCC 27853.
- Klebsiella pneumoniae.

The fungal component will include Candida albicans ATCC 10231.

Additional clinically characterised isolates may be incorporated following institutional approval and appropriate biosafety procedures.

Preliminary Agar-Diffusion Screening

Agar-well or disc-diffusion testing may be employed as a preliminary screening method.

Inhibition-zone diameter will be recorded in millimetres, using appropriate solvent and positive controls.

However, inhibition-zone measurements will not be used as the primary basis for ranking extracts. Plant extracts contain compounds with different molecular sizes, solubilities and diffusion characteristics. Therefore, the absence of a large inhibition zone does not necessarily indicate an absence of antimicrobial activity.

Minimum Inhibitory Concentration Determination

Minimum inhibitory concentrations will be determined using broth microdilution procedures.

For aerobic bacteria, the methodology will follow CLSI M07, 12th edition (Clinical and Laboratory Standards Institute, 2024).

A separate reference methodology will be followed for yeast susceptibility testing, using CLSI M27 where applicable.

The bacterial and fungal protocols must be validated independently because their growth requirements and endpoint interpretations differ.

Serial extract concentrations will be prepared within an experimentally established range. Growth controls, sterility controls, solvent controls and appropriate antimicrobial reference compounds will be included.
The MIC will be defined as the lowest tested extract concentration meeting the prespecified inhibition endpoint.
Plant pigmentation, turbidity and precipitation may interfere with optical or indicator-based measurements. Extract-specific blanks and independent confirmation procedures are therefore essential. Resazurin should not be assumed to be interference-free, particularly when testing strongly reducing plant extracts.

Minimum Bactericidal and Fungicidal Concentrations
Minimum bactericidal concentration will be evaluated through subculture of appropriate non-growth wells onto suitable agar media.
The minimum fungicidal concentration will be determined using an appropriate fungal recovery procedure.
The relationship between MIC and MBC will be examined to distinguish inhibitory effects from bactericidal effects.
A prespecified MBC/MIC ratio may be used as an operational classification criterion, provided that the threshold and interpretation are justified for the organism and assay conditions.

Antimicrobial Breadth
Antimicrobial activity will be analysed across individual microorganisms rather than being reduced immediately to a single overall score.
This distinction is important because an extract may be strongly active against one organism while demonstrating little or no activity against others.
The analysis will therefore distinguish:

- Potency against individual organisms.
- Activity across Gram-positive organisms.
- Activity across Gram-negative organisms.
- Antifungal performance.
- Overall breadth of activity.

Clinical relevance will be discussed cautiously. Experimental MIC values for crude plant extracts cannot automatically be interpreted using clinical susceptibility breakpoints established for conventional antibiotics.

Cytotoxicity and Preliminary Selectivity Assessment
Cytotoxicity will be investigated using a suitable non-malignant mammalian cell model.
The selection of HEK293, Vero or another appropriate cell line should be justified according to the intended biological interpretation and laboratory resources.
A concentration-dependent viability assay will be used to estimate the concentration associated with 50% reduction in cell viability (CC_{50}).
The assay must include cell-only controls, vehicle controls, extract-specific blanks and appropriate reference controls.
The results will be interpreted as preliminary in vitro cytotoxicity rather than comprehensive evidence of human safety.
For each extract–organism combination, a selectivity index will be calculated:

$$SI_{ik}=\frac{CC_{50,i}}{MIC_{ik}}$$

Where:

- \(SI_{ik}\) is the selectivity index of extract \(i\) against organism \(k\).
- \(CC_{50,i}\) is the concentration associated with 50% cytotoxicity.
- \(MIC_{ik}\) is the minimum inhibitory concentration against organism \(k\).

Higher values indicate a greater separation between the concentration associated with antimicrobial inhibition and the concentration associated with mammalian-cell cytotoxicity.
The selectivity index should be interpreted as a preliminary prioritisation measure, not as a clinical therapeutic index.

Statistical Analysis

The statistical framework will distinguish the primary experimental outcomes from exploratory chemical–biological associations.
Descriptive results will be reported using appropriate measures of central tendency and variability.
Experimental replicates will be identified explicitly.
Two-way analysis of variance may be used to examine the effects of plant species, extraction solvent and their interaction, subject to the assumptions of the model.
Where assumptions are not satisfied, suitable alternative approaches or transformed response models will be considered.
Post-hoc comparisons will use appropriate multiplicity correction.
For antioxidant measurements, Pearson or Spearman correlation analysis will examine associations between total phenolic content, total flavonoid content and individual assay outcomes.
Untargeted metabolomics data will be examined using:

- Principal component analysis.
- Hierarchical clustering.
- Differential-feature analysis.
- Feature-based molecular networking.
- Partial least squares regression, where justified.
- Appropriate permutation and validation procedures.

The number of independent biological samples must determine the complexity of predictive models. If the sample size is insufficient for reliable predictive validation, PLS and related models will be presented as exploratory analyses rather than evidence of generalisable prediction.
Multiple comparisons will be controlled using an appropriate false-discovery-rate procedure.
All analytical decisions, exclusions, missing-value treatments and normalisation procedures must be documented. Raw mass-spectrometric data and analysis scripts should be deposited in an accessible repository where permitted.

Novel Conceptual Contribution: Integrated Phytochemical–Bioactivity–Selectivity Framework (IPBSF)

Rationale for Developing the Framework

A recurring weakness in comparative medicinal-plant research is the tendency to rank extracts according to a single experimental outcome.
An extract may demonstrate strong radical-scavenging capacity but weak antimicrobial activity. Another may inhibit bacterial growth at a relatively low concentration but also demonstrate substantial mammalian-cell toxicity. A third may show moderate activity across several organisms while maintaining a more favourable preliminary selectivity profile.
These outcomes cannot be interpreted adequately through a single biological measurement.
The original manuscript addresses this problem through the Integrated Bioactivity Concordance Index. Although the original formulation provides a useful starting point, its mathematical assumptions require further development.

The revised framework separates four dimensions:

1. Chemical characterisation.
2. Antioxidant performance.
3. Antimicrobial potency and breadth.
4. Mammalian-cell selectivity.

The central proposition is that extract prioritisation should reflect the relationship among these dimensions rather than the isolated magnitude of one assay.

Four-Dimensional Analytical Structure

Dimension 1: Chemical characterisation

The chemical dimension includes molecular-feature abundance, metabolite annotations, molecular-network clusters and quantitative marker compounds.

This dimension provides information about the chemical identity and composition of the extract.

Dimension 2: Antioxidant performance

The antioxidant dimension incorporates the normalised outcomes of DPPH, ABTS and FRAP measurements.

The direction of each variable must be standardised so that higher normalised values consistently represent stronger measured antioxidant performance.

For DPPH, the inverse IC_{50} may be used, subject to the appropriate handling of detection limits and non-estimable values.

Dimension 3: Antimicrobial performance

The antimicrobial dimension incorporates MIC measurements across the selected microorganisms. Individual organism-level outcomes should be retained alongside any composite measure. This avoids concealing important differences between microbial groups.

Dimension 4: Preliminary selectivity

The selectivity dimension incorporates the relationship between mammalian-cell cytotoxicity and antimicrobial inhibitory concentrations.

An extract that demonstrates strong antimicrobial activity but poor selectivity should not automatically receive a high prioritisation score.

Mathematical Structure of the Proposed IPBSF

The proposed framework uses three complementary biological dimensions and a separate chemical-concordance analysis.

The antioxidant composite for extract i can be expressed as:

$$A_i=\frac{1}{3}\sum_{j=1}^{3}Z(A_{ij})$$

Where:

- A_i represents the antioxidant composite.
- A_{ij} represents the standardised result of antioxidant assay j.
- Z represents the prespecified normalisation transformation.

The antimicrobial composite can be represented as:

$$M_i=\sum_{k=1}^{K}w_kZ(P_{ik})$$

Where:

- M_i represents the antimicrobial composite.
- P_{ik} represents antimicrobial potency against organism k.
- w_k represents the prespecified weight assigned to organism k.
- The sum of the organism weights equals one.

For MIC-based analysis, the potency transformation must account for the dilution structure, detection limits and censored observations. A logarithmic MIC transformation or a clearly defined rank-based transformation may be preferable to directly calculating reciprocal MIC values.
The safety component can be represented using a bounded transformation:
$$S_{ik}=\frac{Sl_{ik}}{Sl_{ik}+c}$$
Where c is a prespecified scaling constant. The constant must be justified and its influence examined through sensitivity analysis.
A preliminary biological prioritisation score may then be calculated as:
$$B_i=S_i\left[\alpha A_i+(1-\alpha)M_i\right]$$
Where α represents the relative contribution of antioxidant performance to the composite biological score.
However, the chemical dimension must remain analytically separate rather than being inserted into the same equation without a defensible weighting rationale.
Chemical–bioactivity concordance will instead be evaluated through feature-level association, molecular-network interpretation and validated multivariate analysis.
This distinction prevents the framework from treating chemical abundance as equivalent to biological efficacy.

Chemical–Bioactivity Concordance Analysis
The principal novelty of the proposed framework is its emphasis on connecting chemical features with biological outcomes.
The analysis will investigate whether specific molecular features or related molecular-network clusters are associated with:

- Higher antioxidant performance.
- Lower antimicrobial MIC values.
- Greater activity across multiple microorganisms.
- More favourable mammalian-cell selectivity.

A candidate molecular feature should not be designated as an active compound solely because its abundance correlates with a biological measurement.
Such associations may arise from co-occurring metabolites, extraction effects, batch variation or interactions among constituents.
Accordingly, the analysis will distinguish three levels of evidence:
Level 1: Statistical association. A chemical feature demonstrates a reproducible association with a measured biological outcome after appropriate multiple-testing correction.
Level 2: Chemical confirmation. The feature receives stronger structural support through reference standards, additional fragmentation evidence or complementary analytical methods.
Level 3: Functional validation. An isolated or enriched compound demonstrates biological activity through independent experimental testing.
Only the third level provides direct evidence that a particular constituent contributes functionally to the measured activity.

Sensitivity Analysis and Ranking Stability
The relative weights assigned to antioxidant activity, antimicrobial activity and selectivity may influence the final extract ranking.
Therefore, the framework will examine alternative weighting scenarios.
For example, antioxidant and antimicrobial contributions may be evaluated under different values of α, while organism-specific weights may be varied within scientifically justified ranges.
Rank stability can be evaluated using Kendall's rank correlation coefficient.

Where appropriate, bootstrap resampling may be used to estimate uncertainty around the composite rankings.
The final interpretation should identify extracts that remain consistently prioritised across plausible weighting scenarios rather than highlighting only the highest score under one arbitrary set of assumptions.

Why the Revised Framework Offers a Stronger Contribution

The IPBSF improves on conventional screening approaches in five ways.
First, it separates chemical identification from biological performance, avoiding the assumption that a high concentration of a metabolite automatically produces a stronger pharmacological effect.
Second, it integrates multiple antioxidant assays rather than relying on a single chemical reaction.
Third, it distinguishes antimicrobial potency against individual microorganisms from overall antimicrobial breadth.
Fourth, it incorporates preliminary mammalian-cell selectivity to avoid prioritising extracts solely because of strong but potentially non-selective activity.
Fifth, it examines the stability of extract rankings under alternative analytical assumptions.
The framework should be presented as a proposed analytical contribution requiring prospective validation, not as an already validated predictive instrument.

Proposed Results and Reporting Structure

Important scientific qualification: The source manuscript does not contain completed laboratory measurements. Consequently, this section specifies the results required for the completed experimental article. It does not report invented findings.

Extraction Yield and Phytochemical Composition

The first results section should compare extraction yield among the five plant species and three solvent systems.
The analysis should identify whether extraction yield varies significantly according to botanical material, solvent polarity and their interaction.
Extraction yield should not be interpreted as an indicator of biological potency. A high extraction yield may reflect the recovery of abundant inactive constituents, while a lower-yielding fraction may contain a greater concentration of specific active compounds.
The reporting table should contain the following information:

Variable	**Required reporting**
Plant species	Accepted botanical name
Extraction solvent	Hexane, ethyl acetate or water
Extraction yield	Percentage of dry starting material
Total phenolic content	mg GAE/g extract
Total flavonoid content	mg QE/g extract
Biological replicates	Number of independent extractions
Statistical comparison	Effect estimates, confidence intervals and adjusted p-values

Comparative Antioxidant Outcomes

The antioxidant results should be reported independently for DPPH, ABTS and FRAP before the construction of a composite antioxidant measure.
The DPPH results should include concentration–response curves and IC_{50} estimates.
ABTS and FRAP should include calibration information and the appropriate reference-equivalent measurements.
A correlation analysis should determine whether total phenolic and flavonoid measurements adequately explain antioxidant variation.
A weak correlation would not necessarily indicate experimental failure. It could demonstrate that bulk colorimetric measurements do not capture the chemical constituents responsible for the observed antioxidant responses.

LC-MS/MS chemical diversity
The metabolomics section should report the number of reproducibly detected molecular features, quality-control performance, annotation confidence and the distribution of features across botanical species and extraction solvents.
PCA should be used to investigate major chemical differences among the extracts.
Molecular-network analysis should identify related feature clusters and examine whether particular clusters are enriched in specific plant species or solvent fractions.
Confirmed compounds should be distinguished from tentative annotations and unidentified features.

Antimicrobial Activity
The antimicrobial results should provide MIC and MBC/MFC values for each extract–organism combination.
The analysis should identify:

- Extracts demonstrating measurable inhibition.
- Organisms showing comparatively greater susceptibility.
- Differences between Gram-positive and Gram-negative bacteria.
- Differences between bacterial and fungal responses.
- Extracts requiring further investigation because of favourable selectivity.

The results must not describe crude-extract MIC values as equivalent to clinical antibiotic susceptibility categories.

Cytotoxicity and Selectivity
The cytotoxicity section should report CC_{50} estimates, concentration–response curves and organism-specific selectivity indices.
Extracts demonstrating strong antimicrobial activity but poor selectivity should be identified as lower-priority candidates for therapeutic development.
The analysis should also recognise that one mammalian cell line provides only preliminary safety information. Additional cell models and in vivo investigations would be necessary before meaningful conclusions about human safety could be drawn.

Integrated Prioritisation
The final results section should compare rankings obtained through:

1. Antioxidant activity alone.
2. Antimicrobial potency alone.
3. Selectivity alone.
4. The integrated IPBSF.

5. Chemical–bioactivity concordance.

This comparison will demonstrate whether incorporating additional analytical dimensions changes the prioritisation of medicinal-plant extracts.

Discussion

Moving from Descriptive Phytochemical Screening to Integrated Natural-Product Research

The central scientific argument of this research is that the evaluation of medicinal plants should extend beyond the identification of broad phytochemical classes and the measurement of isolated biological responses. Traditional phytochemical screening provides useful preliminary information, but it does not adequately explain why chemically different extracts may demonstrate different biological effects.

Newman and Cragg (2020) and Atanasov et al. (2021) emphasise the continuing relevance of natural products in drug discovery. However, the value of natural-product research depends increasingly on the ability to establish relationships between chemical structure, biological activity and pharmacological suitability.

The proposed framework addresses this requirement by connecting botanical authentication, solvent-dependent extraction, molecular profiling, antioxidant evaluation, antimicrobial susceptibility and preliminary cytotoxicity.

Its importance lies in the recognition that chemical complexity creates both opportunities and methodological difficulties. Crude extracts contain multiple constituents that may interact synergistically, antagonistically or independently. Consequently, the activity of an extract cannot necessarily be attributed to its most abundant constituent.

This perspective supports a transition from descriptive screening towards evidence-based prioritisation of chemical fractions and metabolite families.

The Importance of Extraction-Dependent Chemical Composition

Extraction procedures play a fundamental role in determining which chemical constituents are recovered from medicinal plants.

Alara et al. (2021) discuss the influence of extraction conditions on the recovery of phenolic compounds. Solvent polarity, extraction temperature, duration and processing conditions can influence both extraction efficiency and the stability of recovered compounds.

The proposed comparison of hexane, ethyl acetate and water offers an opportunity to investigate the distribution of chemically different constituents across extraction systems.

Non-polar solvents may favour the recovery of certain lipophilic substances, while intermediate-polarity solvents may recover a different range of phenolic and terpenoid constituents. Aqueous extraction may favour compounds with greater water solubility.

However, solvent categories should not be interpreted as absolute chemical boundaries. Individual compounds exhibit different solubility characteristics, and the chemical composition of an extract depends on several interacting variables.

An important implication is that the biological activity of a medicinal plant should be discussed at the level of the particular extract rather than attributed automatically to the entire species.

For example, a particular fraction of Curcuma longa may contain a higher proportion of curcuminoids, while another extraction procedure may recover a different distribution of constituents. Similar differences may occur between phenolic-rich and terpenoid-rich fractions of Ocimum tenuiflorum.

The proposed study therefore provides a basis for examining whether extraction-dependent chemical variation corresponds to measurable differences in biological activity.

Interpreting Total Phenolic and Flavonoid Measurements

Total phenolic and flavonoid measurements remain useful tools for preliminary comparison, but their interpretative value should not be overstated.

The Folin–Ciocalteu procedure provides an estimate of reducing capacity expressed through a gallic acid calibration. It does not identify individual phenolic compounds or establish that all measured reducing activity originates from phenolics.

Similarly, the aluminium chloride method responds differently to flavonoid structures, meaning that results may not reflect the complete concentration of all flavonoid subclasses.

This limitation has important consequences for comparative medicinal-plant research.

If an extract demonstrates high total phenolic content but relatively weak antioxidant activity in one assay, the finding should not automatically be considered contradictory. Differences in molecular structure, reaction kinetics, solubility and interactions among compounds may explain the outcome.

Conversely, a relatively low total phenolic measurement accompanied by substantial antioxidant activity may indicate the contribution of particular compounds with strong assay responses or the presence of non-phenolic reducing substances.

The proposed integration of colorimetric analysis and LC-MS/MS is intended to improve this interpretation by distinguishing broad chemical estimates from molecular-level evidence.

The Significance of Molecular-Network-Based Chemical Interpretation

Untargeted metabolomics offers a broader understanding of plant chemistry than conventional preliminary screening.

Traditional colorimetric measurements provide aggregate information, whereas LC-MS/MS can generate detailed molecular features that can be compared across extracts.

Feature-based molecular networking further supports the investigation of relationships between structurally related chemical features.

Nothias et al. (2020) demonstrate how feature-based molecular networking can improve the analysis of untargeted mass-spectrometric data through the incorporation of chromatographic information and spectral relationships.

Within the present framework, this approach may help identify groups of related metabolites that occur preferentially in particular plant species or solvent fractions.

Its potential contribution extends beyond the identification of individual compounds. Molecular networks can reveal chemical families whose relative abundance changes alongside biological performance.

Nevertheless, a molecular-network cluster should not be interpreted as proof of a shared pharmacological mechanism. Structural similarity does not necessarily imply identical biological activity.

The most scientifically valuable outcome would be the identification of candidate chemical clusters that demonstrate reproducible associations with specific biological measurements and subsequently withstand independent experimental validation.

Understanding Differences Between Antioxidant Assays

The use of DPPH, ABTS and FRAP is justified by the complementary information provided by these analytical procedures.

Gulcin (2020) and Munteanu and Apetrei (2021) explain the methodological differences among antioxidant assays and the importance of selecting methods appropriate to the research question.
DPPH provides information about radical-scavenging responses under a particular solvent and reaction environment. ABTS offers a complementary radical-cation-based measurement, while FRAP assesses ferric-ion reducing capacity.
Differences between these measurements may reflect the chemical diversity of the extracts.
For instance, catechin-containing fractions of Camellia sinensis may demonstrate a different response pattern from limonoid-associated fractions of Azadirachta indica. Similarly, curcuminoid-containing fractions of Curcuma longa may exhibit different reaction characteristics from phenolic-rich fractions of Moringa oleifera.
These are hypotheses requiring experimental verification, not predetermined comparative findings.
An important implication is that a single antioxidant ranking may conceal meaningful differences between assay mechanisms.
The IPBSF addresses this issue by retaining individual assay outcomes and integrating them only after the direction, scale and limitations of the measurements have been established.
Furthermore, chemical antioxidant capacity must remain conceptually distinct from physiological antioxidant protection. The latter depends on absorption, metabolism, cellular distribution, endogenous antioxidant systems and interactions with biological targets.
Accordingly, the findings of the proposed chemical assays would provide a foundation for subsequent cellular and physiological investigations rather than establish therapeutic efficacy.

Antimicrobial Performance and Microbial Structural Differences

The antimicrobial component of the research addresses whether the chemical diversity of the selected plants corresponds to differences in microbial susceptibility.
Plant-derived compounds may affect microbial membranes, proteins and other cellular processes (Álvarez-Martínez et al., 2021).
However, antimicrobial activity is influenced by several factors, including molecular structure, concentration, microbial physiology, compound solubility and the ability of constituents to reach their cellular targets.
The distinction between Gram-positive and Gram-negative microorganisms provides a useful analytical comparison.
The outer membrane of Gram-negative bacteria can restrict the entry of certain compounds. This may contribute to lower susceptibility to some plant-derived substances.
However, this explanation cannot account for every difference between microbial species. Efflux pumps, metabolic adaptations, membrane composition and other resistance mechanisms may influence susceptibility.
The inclusion of MRSA is particularly important because methicillin resistance represents a clinically significant antimicrobial-resistance phenotype. Nevertheless, an extract demonstrating in vitro activity against MRSA should not automatically be described as a treatment for resistant infections.
Its potential significance depends on the reproducibility of the MIC measurements, the selectivity profile, the chemical identity of the active constituents and subsequent mechanistic investigations.
The use of standardised broth microdilution provides a stronger quantitative basis for comparison than inhibition-zone measurements alone.

The Importance of Cytotoxicity and Preliminary Selectivity

One of the most important improvements over conventional comparative phytochemical screening is the incorporation of mammalian-cell cytotoxicity.
Antimicrobial activity is not sufficient to establish pharmaceutical relevance. A substance that inhibits bacterial growth but also damages mammalian cells may have limited therapeutic value.
The selectivity index provides a preliminary way to distinguish extracts according to the separation between antimicrobial inhibitory concentrations and cytotoxic concentrations.
However, the interpretation of selectivity requires caution.
A favourable selectivity index obtained using one cell line does not establish safety in other tissues. It also does not account for absorption, metabolism, distribution, elimination or possible toxicity associated with long-term exposure.
The proposed framework therefore treats cytotoxicity as a prioritisation criterion rather than a definitive safety assessment.
This is a particularly important conceptual improvement because it shifts the research question from identifying the most biologically active extract to identifying extracts that combine measurable activity with a more favourable preliminary selectivity profile.

Why Integrated Ranking May Improve Medicinal-Plant Prioritisation

Conventional comparative studies frequently identify the strongest extract according to a single biological measurement.
Such rankings may be misleading because different biological dimensions are not interchangeable.
Consider three hypothetical extracts:

- Extract A demonstrates strong antioxidant performance but weak antimicrobial activity.
- Extract B demonstrates relatively strong antimicrobial inhibition but also substantial cytotoxicity.
- Extract C demonstrates moderate antioxidant and antimicrobial performance alongside a more favourable selectivity profile.

A single-assay ranking might identify Extract A or B as superior, depending on the selected outcome. However, a multi-dimensional prioritisation framework may identify Extract C as a more appropriate candidate for subsequent investigation.
This example illustrates the rationale behind the IPBSF. Its purpose is not to establish that moderate activity is always preferable to high activity, but to make the criteria for prioritisation explicit.
The sensitivity analysis is equally important. If the ranking changes dramatically when the weighting coefficient is adjusted slightly, the apparent superiority of an extract is not robust.
A scientifically useful prioritisation framework should identify candidates whose relative performance remains reasonably stable across defensible analytical assumptions.

Implications for Natural-Product Drug Discovery

The proposed framework provides a structured pathway for selecting extracts and chemical families for subsequent investigation.
Extracts demonstrating promising biological performance and favourable selectivity may be prioritised for bioassay-guided fractionation.
Subsequent research should involve:

1. Fractionation using suitable chromatographic techniques.
2. Repeated biological testing of individual fractions.
3. Isolation of candidate compounds.
4. Structural elucidation using NMR and high-resolution mass spectrometry.
5. Confirmation of compound identity using authentic standards.

6. Independent testing of isolated compounds.
7. Investigation of possible synergistic and antagonistic interactions.
8. Mechanistic assessment of antimicrobial effects.
9. Cellular antioxidant and toxicity experiments.
10. Appropriate in vivo evaluation before any therapeutic claims are considered.

The proposed approach also provides opportunities for investigating interactions between plant-derived compounds and conventional antimicrobial agents.

However, combination studies should be designed carefully, using appropriate checkerboard or time-kill methods, rather than assuming that the combination of two active substances will necessarily produce synergy.

Broader Significance for Medicinal-Plant Research

The contribution of this research extends beyond the five selected species.

Its underlying analytical principles can be applied to other medicinal plants, botanical preparations and natural-product mixtures.

The central methodological proposition is that medicinal-plant investigations should integrate botanical identity, chemical characterisation, biological measurement and safety assessment.

Heinrich et al. (2022) emphasise the importance of standardised reporting in phytochemical research. The present framework builds upon this principle by connecting chemical documentation with biological prioritisation.

Such integration may improve reproducibility, facilitate comparison between laboratories and support more efficient selection of candidates for advanced pharmacological investigation.

Conclusion

This research develops an integrated comparative framework for investigating the phytochemical composition, antioxidant capacity, antimicrobial performance and preliminary selectivity of five medicinal plants: Azadirachta indica, Ocimum tenuiflorum, Curcuma longa, Moringa oleifera and Camellia sinensis.

Its principal contribution is the integration of standardised extraction, quantitative phytochemical analysis, untargeted LC-MS/MS profiling, feature-based molecular networking, complementary antioxidant measurements, antimicrobial susceptibility assessment and mammalian-cell cytotoxicity evaluation.

The proposed Integrated Phytochemical–Bioactivity–Selectivity Framework addresses an important limitation of conventional screening studies: the tendency to prioritise extracts according to isolated biological outcomes without considering their chemical identity, activity breadth and preliminary safety characteristics.

By separating chemical evidence from biological performance and examining their relationships through statistical analysis, the framework offers a more transparent approach to identifying promising extracts and metabolite families.

Importantly, the research does not assume that any of the five plants will necessarily demonstrate superior biological activity. Their comparative performance must be established through properly controlled experiments.

The proposed methodology provides a foundation for subsequent bioassay-guided fractionation, compound isolation, structural confirmation and mechanistic investigation. Ultimately, its value will depend on experimental reproducibility, independent validation and the ability to translate preliminary laboratory observations into scientifically supported natural-product leads.

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