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MEDICINAL PLANTS AND PHYTOCHEMICAL DIVERSITY: CHARACTERIZATION OF BIOACTIVE COMPOUNDS AND THEIR BIOLOGICAL ACTIVITIES

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Abstract

Plant secondary metabolites represent an evolutionary adaptation enabling flora to withstand abiotic stress, defend against pathogens, and mediate ecological interactions. These specialized compounds including polyphenols, flavonoids, alkaloids, terpenoids, saponins, steroids, and glycosides exhibit extraordinary structural diversity with significant pharmacological potential. This review critically evaluates the ecological and biogeographical drivers of phytochemical variation, contrasts conventional extraction protocols with Natural Deep Eutectic Solvent (NADES)-based green extraction technologies, outlines advanced spectroscopic characterization methodologies, and details pharmacological mechanisms underlying antibacterial, antioxidant, cytotoxic, and anti-inflammatory activities. Phytochemical composition is dynamically regulated by genetic factors and environmental conditions, with abiotic stress such as UV radiation, water deficits, and temperature fluctuations upregulating specific secondary metabolic pathways. Geographic origin and tissue-specific gene expression further influence metabolite accumulation, as demonstrated in Prosopis farcta, Azadirachta indica, and Reynoutria species. Conventional extraction methods including Soxhlet and maceration suffer from thermal degradation, toxic solvent consumption, and low selectivity. NADES combined with Ultrasound-Assisted Extraction offers sustainable alternatives, achieving 2- to 6-fold higher bioactive recovery at mild temperatures (≤40°C) while protecting labile compounds. Advanced analytical platforms UPLC-HRMS, NMR spectroscopy, and Feature-Based Molecular Networking through GNPS have accelerated dereplication and novel compound identification. Pharmacological evaluations demonstrate that phenolics, terpenoids, and alkaloids disrupt bacterial membranes (MIC 0.06–0.50 mg/mL), neutralize reactive oxygen species via Hydrogen Atom Transfer and Single Electron Transfer mechanisms, induce apoptosis in cancer cell lines, suppress NF-κB-mediated inflammation, and inhibit α-glucosidase and α-amylase for diabetes management. However, methodological heterogeneity, standardization bottlenecks, NADES industrial scale-up challenges, and spectral database limitations constrain clinical translation. Integrating machine learning algorithms and expanded spectral libraries promises to bridge unannotated "dark matter" gaps in phytochemical discovery.

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1. INTRODUCTION

Plant secondary metabolites represent an evolutionary mechanism for survival, enabling flora to adapt to abiotic stress, defend against pathogen infection and herbivory, and signal ecological interactions. Unlike primary metabolites, which are essential for basic cellular growth and reproduction, specialized secondary metabolites exhibit extraordinary structural diversity. These chemical architectures are broadly categorized into polyphenols, flavonoids, alkaloids, terpenoids, saponins, steroids, and glycosides (Wink, 2010). In rural and resource-constrained regions globally, traditional plant-based pharmacopoeias continue to serve as the foundation of primary healthcare, with over 80% of populations relying on botanical remedies (WHO, 2013).

The transition from empirical ethnobotanical knowledge to evidence-based phytopharmacology requires rigorous scientific characterization (Heinrich et al., 2020). Historically, natural product discovery was constrained by crude extraction techniques, low-resolution separation, and tedious isolated-compound testing. Modern phytochemistry integrates sustainable extraction platforms with high-resolution analytical instrumentation and computational metabolomics (Sumner et al., 2007). Couplings such as Ultra-Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry (UPLC-HRMS), Nuclear Magnetic Resonance (NMR) spectroscopy, and MS/MS-based molecular networking through platforms like the Global Natural Products Social Molecular Networking (GNPS) ecosystem have accelerated dereplication and novel compound identification (Wang et al., 2016).

This review provides a critical evaluation of plant secondary metabolite diversity. It examines the ecological and biogeographical drivers of phytochemical variation, contrasts conventional extraction protocols with Natural Deep Eutectic Solvent (NADES)-based green extraction technologies, outlines advanced spectroscopic characterization methodologies, and details the pharmacological mechanisms underlying observed antibacterial, antioxidant, cytotoxic, and anti-inflammatory activities (Newman & Cragg, 2020).

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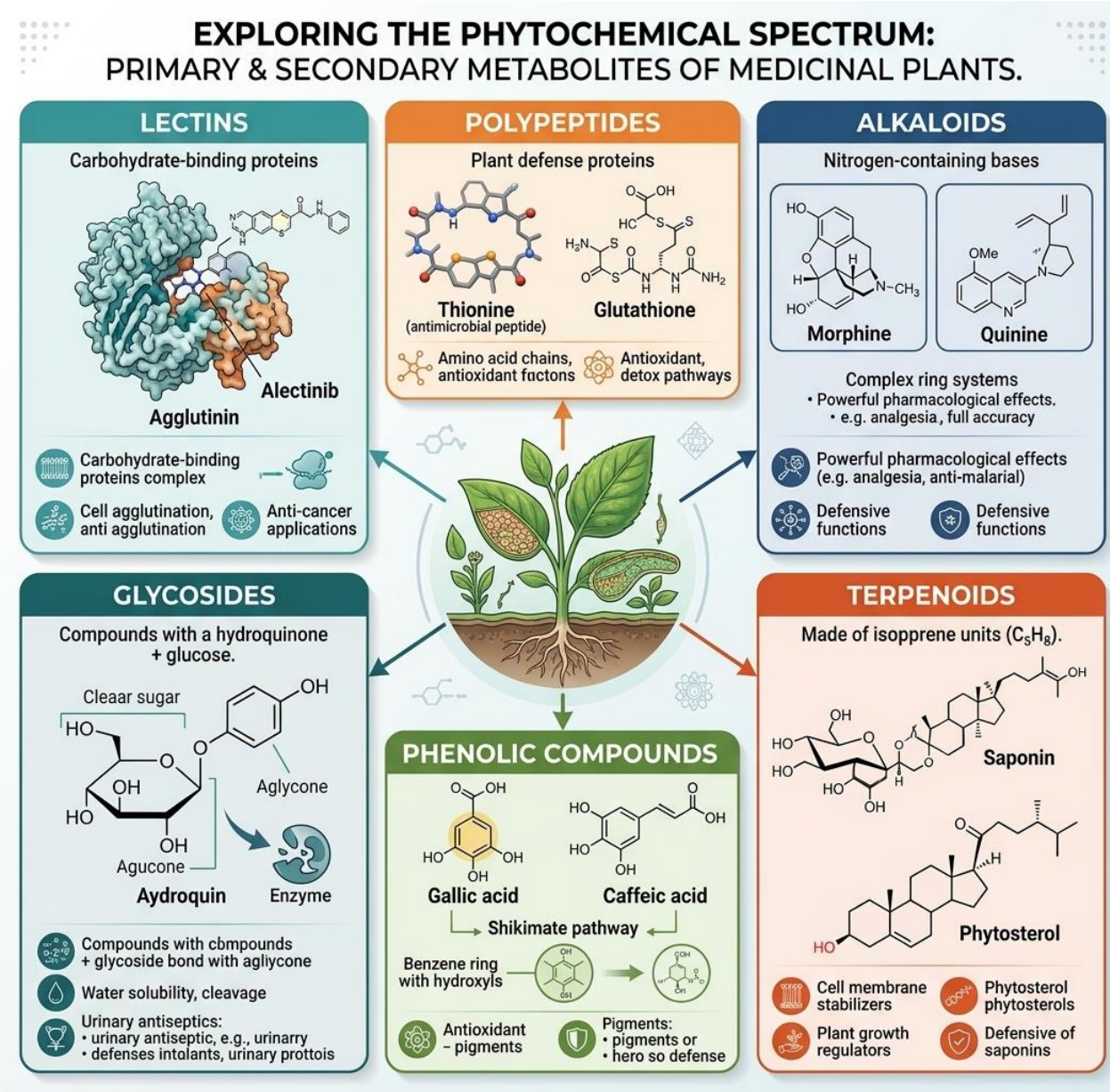


Figure 1. Structural Diversity, Chemical Classification, and Functional Biosynthetic Pathways of Primary and Secondary Plant Metabolites.

2. Chemotaxonomy, Biogeography, and Ecological Drivers of Phytochemical Diversity

2.1. Abiotic Stress and Metabolic Adaptation

Phytochemical composition is dynamically regulated by genetic factors and environmental conditions. Plants exposed to severe abiotic stress such as intense ultraviolet (UV) radiation, prolonged water deficits, extreme temperature fluctuations, and specialized geological substrates upregulate specific secondary metabolic pathways to protect their cellular machinery (Hartmann, 2007).

In mountain ecosystems such as the Pindus range in Greece, altitudinal gradients and microclimatic variations create localized ecological niches. These environmental pressures induce oxidative stress, triggering the accumulation of photoprotective and radical-scavenging secondary metabolites,

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particularly polyphenols, flavonoids, and terpenoids. Consequently, distinct chemotypes develop within the same botanical species across geographical gradients, directly altering the therapeutic potential and antioxidant capacity of plant extracts (Wink, 2008).

2.2. Geographic Differentiation and Organ Specificity

The expression of secondary metabolites is controlled by both geographic origin and tissue-specific gene expression. Organ-specific metabolic partitioning ensures that specialized compounds accumulate where defense or physiological regulation is most needed (Kessler & Baldwin, 2002).

A clear example of this spatial and organ-specific differentiation is seen in *Prosopis farcta* (Fabaceae), a medicinal species distributed across arid and semi-arid regions of North Africa and the Middle East. Comprehensive profiling of *P. farcta* across Egyptian populations revealed 14 pure isolated compounds and 72 distinct metabolites characterized via HPLC-PDA-ESI-MS/MS. In rhizomes and roots, specific phenolic acids and flavonoid glycosides accumulate to counter subterranean oxidative stress and soil microbial challenges. In contrast, Tunisian populations exhibit distinct organ-specific variations, yielding 16 dominant phenolic markers distributed across leaves, stems, and fruits (Wolfender et al., 2009).

Similarly, meta-analyses of medicinal species across Sudan encompassing Sahelian, Afro-tropical, and Arabian floristic zones demonstrate that geographical isolation and environmental conditions yield distinct chemotaxonomic clusters. Analyzing these biogeographical and tissue-level patterns is essential for bioprospecting, as harvesting specific plant parts at optimal geographical locations maximizes bioactive yields (Wolfender et al., 2015).

Table 1: Biogeographical and Chemotaxonomic Profiling of Selected Medicinal Flora

Botanical Species	Botanical Family	Primary Geographic Origin / Ecological Zone	Key Secondary Metabolite Classes	Representative Identified Bioactive Compounds	Primary Target Biological Activities
Azadirachta indica A. Juss. (Neem)	Meliaceae	West Africa (e.g., Chad), South Asia	Phthalate esters, Terpenoids, Flavonoids, Saponins, Alkaloids	Diethyl phthalate, Phytol, Quercetin derivatives, Nimbin	Antibacterial, Antioxidant, Insecticidal
Prosopis farcta (Banks & Sol.)	Fabaceae	Arid & Semi-Arid North Africa (Egypt, Tunisia), Middle East	Phenolics, Flavonoid glycosides, Tannins, Phytosterols	Quercetin-3-O-glucoside, Luteolin derivatives, Gallic acid	Cytotoxic, Antioxidant, Anti-diabetic, Anti-inflammatory
Reynoutria species (*R. japonica*, *R. sachalinensis*)	Polygonaceae	Temperate Eurasia, Mountainous regions	Anthraquinones, Stilbenes, Phenolic acids	Resveratrol, Polydatin, Emodin, Physcion	Antioxidant, Anti-inflammatory, Antimicrobial
Mediterranean Woody Flora (*Pindus System*)	Multiple	Mediterranean Basin (High-altitude	Flavonoids, Phenolic acids, Essential oil Terpenes	Baicalein, Scutellarein, Wogonin, Oroxylin A	Photoprotective, Antioxidant, Cytotoxic

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		limestone, Greece)			

3. Conventional and Emerging Green Extraction Methodologies

3.1. Fundamentals and Limitations of Conventional Extraction

The extraction of bioactive compounds from plant matrices is the initial step in natural product characterization. Solute recovery depends on solvent polarity, plant tissue particle size, solid-to-solvent ratio, temperature, and agitation. Conventional methods including Soxhlet extraction, room-temperature maceration, reflux, and hydrodistillation rely heavily on organic solvents such as methanol, ethanol, hexane, chloroform, and diethyl ether (Dai et al., 2013).

In Soxhlet extraction, organic solvents are continuously boiled and condensed through dried plant material. While Soxhlet processing achieves high crude extract yields, prolonged exposure to elevated temperatures causes thermal degradation of heat-sensitive compounds, including certain anthocyanins, flavonoids, and volatile terpenoids. Furthermore, conventional extraction requires large volumes of toxic, volatile organic solvents (VOCs), creating environmental, health, and economic drawbacks (Choi et al., 2011).

3.2. Green Extraction Paradigm: Natural Deep Eutectic Solvents (NADES)

To address these limitations, natural product research has increasingly adopted green extraction technologies. Natural Deep Eutectic Solvents (NADES) are sustainable alternatives to conventional organic solvents. NADES are liquid mixtures formed by combining hydrogen bond acceptors (HBA), such as choline chloride, with hydrogen bond donors (HBD), such as organic acids (citric acid, malic acid), sugars (glucose, sucrose), or polyols (glycerol, urea), at specific molar ratios (Dunn & Ellis, 2005).

The resulting supramolecular hydrogen-bonding structure lowers the melting point below that of the individual components, yielding a stable liquid at room temperature. NADES offer customizable polarity, negligible vapor pressure, biodegradability, non-toxicity, and high solubilization capacity for both polar and moderately non-polar biomolecules. Furthermore, the supramolecular hydrogen-bond network of NADES protects labile antioxidants, pigments, and polyphenols from oxidative and thermal degradation during processing (Atanasov et al., 2021).

A key physical limitation of pure NADES is high viscosity, which impedes mass transfer and solute diffusion. Adding controlled amounts of water typically 20% to 40% w/w hydration significantly reduces viscosity and surface tension, increasing mass transfer rates while maintaining the supramolecular eutectic structure (Lee et al., 2026).

3.3. Process Intensification and Solvent Recyclability

Combining NADES with emerging technologies such as Ultrasound-Assisted Extraction (UAE) or Microwave-Assisted Extraction (MAE) provides synergistic improvements in efficiency. UAE uses acoustic cavitation the formation, growth, and collapse of microbubbles within the liquid medium to generate localized high pressures and micro-jets. This process ruptures plant cell walls, speeding solvent penetration and solute dissolution (Shafiq et al., 2026).

Optimized NADES-UAE protocols operate at mild temperatures ($\leq 40°C$) and short extraction times (≤ 50 min), preventing the thermal degradation seen in traditional Soxhlet methods. For instance, extracting flavonoids (such as baicalein, scutellarein, wogonin, and oroxylin A) from *Ipomoea pes-caprae* leaves using choline chloride-based NADES combined with UAE produced bioactive yields 2- to 6-fold higher than conventional aqueous methanol. In addition, these NADES systems can be recovered and recycled across multiple extraction cycles with minimal loss in total flavonoid recovery, supporting green engineering principles (Abdelrahman et al., 2023).

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Table 2: Comparative Analysis of Extraction Methodologies for Bioactive Compound Recovery

Extraction Methodology	Typical Solvents / Reagents	Operating Temperature & Duration	Recovery Yield & Selectivity	Energy & Environmental Impact	Primary Limitations
Soxhlet Extraction	Ethanol, Methanol, Water, Chloroform, Hexane	60–100°C; 4–24 hours	Exhaustive yield for heat-stable compounds; low selectivity	High energy usage; high organic solvent consumption and waste	Thermal degradation of labile compounds; toxic solvent residue
Maceration	Hydroalcoholic mixtures, Distilled Water	20–25°C (Ambient); 24–72 hours	Moderate yield; non-selective crude extraction	Low energy usage; high solvent volume requirements	Slow mass transfer; incomplete extraction; high solvent consumption
NADES + UAE (Green Platform)	Choline Chloride + Glycerol / Urea / Citric Acid (20–40% H_2O)	≤ 40°C; ≤ 50 minutes	2–6 × higher recovery of bioactives vs. organic solvents; high protection of labile molecules	Low energy consumption; zero VOC emission; biodegradable and recyclable solvent	High initial viscosity requiring precise hydration tuning; complex downstream recovery
Microwave-Assisted Extraction (MAE)	Water, Hydroalcoholic, NADES systems	50–80°C; 5–20 minutes	High recovery of polar and moderately polar bioactives	Energy efficient due to rapid localized heating	Risk of localized thermal hotspots; restricted to polar, microwave-absorbing matrices

4. Advanced Analytical Characterization and Metabolomics Techniques

4.1. Chromatographic Separation and High-Resolution Mass Spectrometry

Accurate profiling of complex phytochemical mixtures requires high-resolution separation platforms coupled with sensitive detection systems. High-Performance Liquid Chromatography coupled with Photodiode Array Detection (HPLC-DAD) and Ultra-Performance Liquid Chromatography (UPLC) provide baseline separation and UV/Vi's characterization of phenolics, flavonoids, and alkaloid markers (Savic et al., 2026).

Coupling liquid chromatography with High-Resolution Tandem Mass Spectrometry (LC-HRMS), such as Quadrupole Time-of-Flight (LC-ESI-QTOF-MS/MS) systems, enables exact mass measurements with sub-part-per-million (ppm) error rates. This high mass accuracy allows determination of elemental compositions (molecular formulas) and fragmentation pathways for unknown peaks (Locatelli et al., 2012).

For example, characterization of ethanol extracts of *Azadirachta indica* leaves grown in Chad using

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HPLC-MS identified the major organic constituent as diethyl phthalate (92.31%), alongside minor terpenes like phytol (1.68%) and various polyphenolic derivatives. Similarly, LC-ESI-MS/MS analysis of *Reynoutria* species enabled simultaneous quantification and structural characterization of diverse anthraquinone aglycones and stilbene glycosides (Custodio-Mendoza et al., 2024).

4.2. Nuclear Magnetic Resonance (NMR) Structural Elucidation

While mass spectrometry provides mass-to-charge ratios and fragmentation patterns, unambiguous structural assignments including regiochemistry, stereochemistry, and complex glycosidic link configurations depend heavily on Nuclear Magnetic Resonance (NMR) spectroscopy. One-dimensional proton (image) and carbon (image) NMR experiments yield basic chemical shift profiles. However, complex secondary metabolites require two-dimensional (2D) homonuclear and heteronuclear NMR correlation techniques to resolve overlapping signals (Huang et al., 2024).

Homogeneous correlation spectroscopy (COSY) maps spin-spin couplings between adjacent protons, establishing contiguous proton coupling networks. Heteronuclear single quantum coherence (HSQC) correlates protons directly to their attached carbon atoms through one-bond scalar couplings (image), identifying methyl, methylene, and methine carbons (Pauli et al., 2005). To link isolated spin systems across heteroatoms or quaternary carbons, heteronuclear multiple bond correlation (HMBC) captures long-range proton-carbon couplings across two or three bonds (image and image). Spatial proximity and relative stereochemistry are further resolved using Transverse Rotating Frame Overhauser Effect Spectroscopy (TROESY) or Nuclear Overhauser Effect Spectroscopy (NOESY) (Claridge, 2016). Hyphenated pseudo-LC-NMR protocols allow structural assignment of microgram-quantity metabolites directly from crude or fractionated extracts without requiring extensive physical isolation (Menard & Schug, 2025).

4.3. Feature-Based Molecular Networking (FBMN) and GNPS Workflows

Untargeted LC-MS/MS experiments generate massive, highly complex datasets. Manual spectral interpretation is slow and limited by the availability of pure reference standards. To overcome these challenges, tandem mass spectrometry molecular networking integrated within the Global Natural Products Social Molecular Networking (GNPS / GNPS2) infrastructure has emerged as a key approach for high-throughput dereplication (Novoa-Carballal et al., 2011).

The methodology operates on the structure-spectrum correlation principle, which states that chemical structural similarity produces analogous MS/MS gas-phase fragmentation patterns. In this computational workflow, raw LC-HRMS/MS data are preprocessed using feature alignment algorithms such as MZmine or XCMS to extract accurate mass-to-charge ratios, retention times, and integrated peak areas. The aligned spectra are uploaded to GNPS, where mathematical algorithms such as modified cosine similarity scores, entropy similarity, Spec2Vec, or MS2DeepScore calculate pairwise spectral similarity matrix vectors (Elkins et al., 2019).

The resulting data are visualized as graphical networks where individual precursor ion features are displayed as nodes, and structural similarity connections are drawn as edges. Node sizes scale with relative chromatographic abundance, while edge thickness reflects modified cosine similarity scores. Concurrently, GNPS searches experimental spectra against public spectral libraries. Matches serve as "seed" nodes within structural clusters, allowing researchers to propagate annotations across connected, unannotated neighbor nodes. Feature-Based Molecular Networking (FBMN) and Ion Identity Molecular Networking (IIMN) streamline this process by resolving chromatographic isomers, collapsing adduct variations, and linking uncharacterized structural analogues (Martin et al., 2002).

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Table 3: Spectroscopic and Spectrometric Platforms in Natural Product Dereplication and Structural Elucidation

Analytical Platform	Instrumentation Coupling / Informatics	Primary Analytical Application	Resolution & Data Output	Primary Role in Phytochemical Discovery
UPLC-DAD / HPLC-UV	Photodiode Array Detection	Quantitative profiling & chromophore identification	Chromatographic retention time (t_R); UV λ_{max} spectra	Target compound quantification and quality control standardization
LC-ESI-QTOF-MS/MS	High-Resolution Electrospray Ionization MS	Mass determination & tandem fragmentation	Accurate mass (m/z, < 5 ppm error); MS2 fragment spectra	Molecular formula prediction and high-throughput dereplication
NMR Spectroscopy	^{1}H, ^{13}C, COSY, HSQC, HMBC, TROESY	Regiochemical & stereochemical elucidation	Chemical shifts (δ, ppm); scalar coupling constants (J, Hz)	Definite, unambiguous 3D structural assignment of isolated molecules
Molecular Networking	FBMN / IIMN on GNPS & GNPS2 Ecosystem	Global chemical space visual clustering	Pairwise Cosine/Spec2Vec scores; network cluster graphs	Automated dereplication, structural analogue tracking, and dark matter discovery

5. Biological Activities and Pharmacological Mechanisms of Action

5.1. Antimicrobial and Antibacterial Potency

Phytochemicals exhibit antimicrobial activity through multiple targeted cellular mechanisms. Phenolics, terpenoids, and alkaloids disrupt bacterial outer membrane integrity, collapse trans-membrane proton gradients, inhibit cell wall synthesis, and block essential metabolic enzymes (Rafeeq et al., 2025).

Standardized antibacterial evaluations measure the Minimum Inhibitory Concentration (MIC) the lowest compound concentration that prevents visible bacterial growth and the Minimum Bactericidal Concentration (MBC). Hydroalcoholic and leaf extracts of *Azadirachta indica* show strong antibacterial activity against Gram-positive and Gram-negative pathogens, with MIC values ranging from 0.06 to 0.50 mg/mL (Radulovic et al., 2013).

The elevated antibacterial activity observed in ethanol and methanol fractions correlates with high concentrations of lipophilic terpenes (e.g., phytol) and phenolic derivatives, which penetrate bacterial lipid bilayers and cause intracellular leakage (Ghosh et al., 2025).

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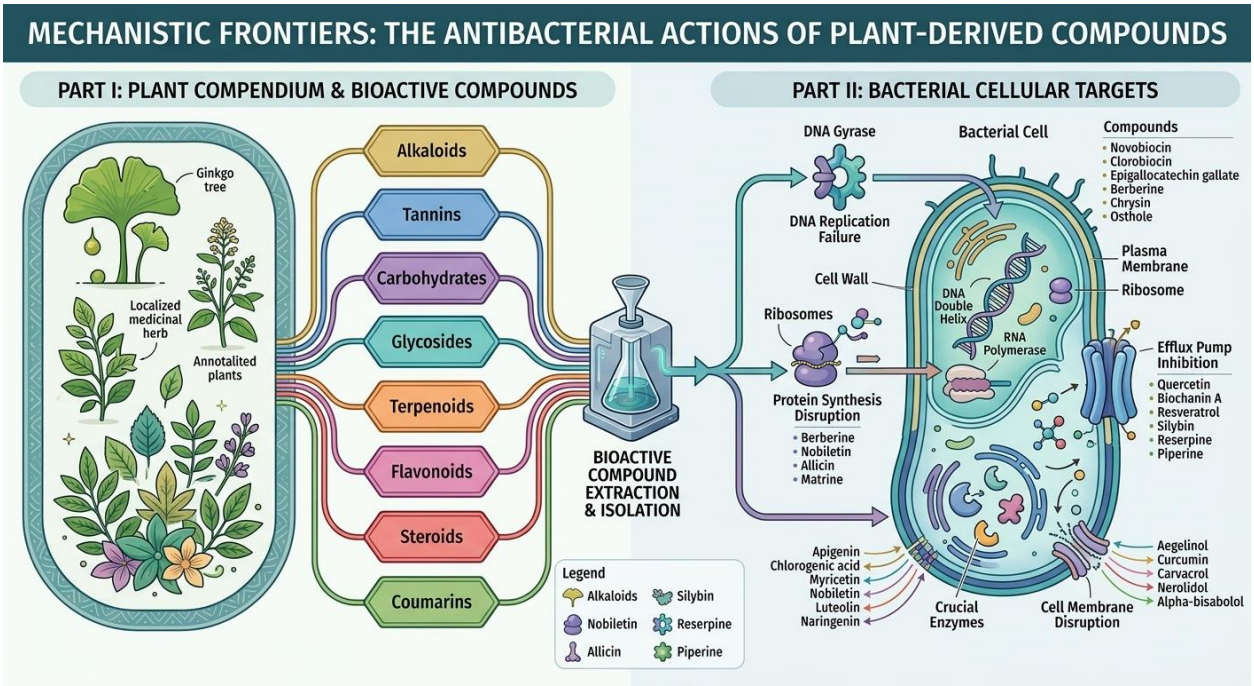


Figure 1. Comprehensive Framework of Botanical Bioactive Compound Classes and Their Mechanistic Antibacterial Targets within Bacterial Cells.

5.2. Antioxidant and Radical Scavenging Mechanisms

Endogenous oxidative stress driven by excessive production of reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\cdot}$), superoxide anions ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2) causes lipid peroxidation, protein oxidation, and DNA cleavage. Plant secondary metabolites neutralize ROS through two primary chemical pathways: Hydrogen Atom Transfer (HAT), where the antioxidant donates a hydrogen atom to stabilize free radicals, and Single Electron Transfer (SET), where the antioxidant transfers an electron to reduce radical species, forming a stable, resonance-stabilized phenoxyl radical (Sies & Jones, 2020).

Phenolic acids, flavonoids, and condensed tannins act as radical scavengers and metal chelators, binding transition metal ions (Fe^{2+}, Cu^{2+}) to prevent Fenton reaction-driven hydroxyl radical generation. For example, ethyl acetate and *n*-butanol fractions of *Prosopis farcta* roots, rich in quercetin glycosides and gallic acid derivatives, display strong DPPH and ABTS radical scavenging capacity. Similarly, polyphenol enrichment in *Reynoutria* species correlates with strong antioxidant capacity, offering cell protection against oxidative stress (Sikder et al., 2025).

5.3. Cytotoxic, Anti-inflammatory, and Anti-diabetic Properties

Beyond antimicrobial and antioxidant roles, plant secondary metabolites modulate key physiological and signaling pathways. In cytotoxic and anticancer contexts, bioactive fractions rich in specific polyphenols, alkaloids, and saponins induce apoptosis in cancer cell lines by triggering cell cycle arrest at G0/G1 or G2/M phases, activating pro-apoptotic caspase cascades, upregulating Bax expression, and downregulating anti-apoptotic Bcl-2 proteins (Bhatti et al., 2022).

Regarding anti-inflammatory actions, flavonoid aglycones such as baicalein and wogonin suppress nuclear factor kappa B (NF-κB) translocation. This suppresses inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression, reducing pro-inflammatory prostaglandin (PGE_2) and cytokine release ($TNF\text{-}\alpha$, $IL\text{-}6$) (Zasheva et al., 2024).

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In anti-diabetic pathways, polyphenol-rich plant extracts lower postprandial hyperglycemia by inhibiting key carbohydrate-hydrolyzing enzymes, specifically α-glucosidase and α-amylase. Inhibiting these enzymes delays intestinal carbohydrate digestion and glucose absorption, presenting a therapeutic target for managing type 2 diabetes mellitus (Bansal et al., 2026).

Table 4: Key Bioactive Classes, Representative Compounds, and Pharmacological Mechanisms

Phytochemical Class	Representative Bioactive Compounds	Cellular & Molecular Mechanism of Action	Assayed Biological Activity	Quantitative Pharmacological Endpoints
Flavonoids & Phenolic Acids	Quercetin, Chlorogenic acid, Baicalein, Gallic acid	Radical neutralization via HAT/SET; NF-κB pathway suppression; α-glucosidase inhibition	Antioxidant, Anti-inflammatory, Anti-diabetic	High DPPH/ABTS scavenging ($IC_{50} <$ 10 μg/mL); reduced PGE_2 production
Terpenoids & Phthalate Derivatives	Phytol, Nimbin, Diethyl phthalate, Limonoids	Destabilization of bacterial cell membranes; induction of mitochondrial apoptosis pathways	Antibacterial, Cytotoxic, Insecticidal	Antibacterial MIC values 0.06–0.50 mg/mL; cancer cell growth inhibition
Anthraquinones & Stilbenes	Emodin, Physcion, Resveratrol, Polydatin	Scavenging ROS; regulating SIRT1/AMPK signaling pathways; blocking COX-2 expression	Anti-inflammatory, Cytotoxic, Cardioprotective	Downregulation of pro-inflammatory cytokines (TNF-α, IL-1β)
Saponins & Steroids	Diosgenin, Saponin aglycones	Permeabilization of cellular lipid membranes; caspase-3/9 activation; cell cycle arrest	Cytotoxic, Anthelmintic, Immunomodulatory	Dose-dependent cytotoxicity against malignant tumor cell lines

6. Challenges, Regulatory Perspectives, and Future Horizons

6.1. Methodological Heterogeneity and Standardization Bottlenecks

A significant challenge in natural product research is methodological heterogeneity. Variations in solvent polarity, extraction techniques, thermal profiles, plant tissue selection, and seasonal harvesting times create batch-to-batch variation in secondary metabolite concentrations. This inconsistency causes wide variability in reported quantitative IC_{50} values and antimicrobial MIC endpoints across different studies (Huang et al., 2024).

Addressing this requires establishing standardized analytical workflows. Implementing validated marker-based HPLC/LC-MS quantitative protocols alongside strict Good Agricultural and Collection Practices (GACP) is essential for ensuring batch-to-batch reproducibility and safety in commercial phytomedicines (Savic et al., 2026).

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6.2. Industrial Scale-Up of NADES and Regulatory Clearance

Transitioning green extraction systems specifically Natural Deep Eutectic Solvents (NADES) from laboratory scale to industrial production presents engineering and regulatory challenges. High solvent viscosity requires specialized high-pressure pumping systems, precise temperature regulation, and controlled hydration tuning (20–40% H_2O). Furthermore, isolating target bioactives from non-volatile NADES media requires optimized downstream recovery protocols, such as macroporous resin adsorption, liquid-liquid back-extraction, or anti-solvent precipitation (Custodio-Mendoza et al., 2024).

From a regulatory perspective, applying NADES-derived extracts in food, nutraceutical, and pharmaceutical formulations requires formal Generally Recognized as Safe (GRAS) status, thorough *in vivo* toxicological profiling, and comprehensive lifecycle assessments to confirm overall safety Kessler & Baldwin, 2002).

6.3. Machine Learning and Spectral Database Expansion

While MS/MS molecular networking has accelerated natural product dereplication, spectral matching remains constrained by public database coverage. A substantial portion of the plant chemical space consists of unannotated "dark matter" nodes lacking reference spectra in repositories like GNPS, MassBank, or HMDB (Wink, 2008).

Integrating machine learning (ML) algorithms, deep learning spectral predictors (e.g., SIRIUS, CANOPUS, MS2DeepScore, Spec2Vec), and automated structure-elucidation pipelines into untargeted metabolomics workflows helps bridge this gap. Training computational models on large biosynthetic networks enables automated prediction of compound classes, functional group modifications, and core scaffold structures directly from unannotated fragmentation spectra (Heinrich et al., 2020).

Conclusion

This review demonstrates that plant secondary metabolites constitute a structurally diverse and pharmacologically significant reservoir for therapeutic development. The synthesis of ecological, biogeographical, and tissue-specific factors reveals that phytochemical diversity is not random but represents a finely tuned evolutionary response to environmental pressures. Chemotaxonomic profiling across species such as *Prosopis farcta*, *Azadirachta indica*, and *Reynoutria* species confirms that geographical isolation and organ-specific expression yield distinct chemical phenotypes with direct implications for bioprospecting and optimal harvesting strategies. The transition from conventional extraction methodologies to NADES-based green platforms represents a paradigm shift in natural product research. NADES systems offer customizable polarity, biodegradability, and superior protection of labile bioactives, with optimized NADES-UAE protocols achieving 2- to 6-fold higher yields while operating at mild temperatures. However, industrial scale-up requires addressing viscosity limitations, downstream recovery complexity, and regulatory clearance through GRAS status and comprehensive toxicological profiling. Advanced analytical platforms UPLC-HRMS, multidimensional NMR, and GNPS-based molecular networking have revolutionized dereplication capabilities, enabling rapid annotation of known compounds and targeted discovery of novel analogues. These tools, combined with machine learning spectral predictors, are progressively illuminating the "dark matter" of unannotated phytochemical space. Pharmacological investigations confirm that flavonoids, phenolic acids, terpenoids, anthraquinones, stilbenes, and saponins exert antibacterial, antioxidant, cytotoxic, anti-inflammatory, and anti-diabetic activities through defined molecular mechanisms including membrane disruption, ROS neutralization via HAT/SET pathways, NF-κB suppression, caspase activation, and enzyme inhibition. Quantitative endpoints such as MIC values (0.06–0.50 mg/mL), IC_{50} values (<10 μg/mL for DPPH/ABTS), and cytokine downregulation provide benchmarks for standardization. Despite these advances, methodological heterogeneity, batch-to-batch variability, and lack of validated

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marker-based quality control protocols impede clinical translation. Future research must prioritize standardized extraction and analytical workflows, GACP implementation, comprehensive *in vivo* efficacy and safety studies, and integration of computational metabolomics with experimental validation. By bridging ethnobotanical knowledge with evidence-based phytopharmacology, the field can realize the full therapeutic potential of plant secondary metabolites for global health challenges including antimicrobial resistance, oxidative stress-related diseases, cancer, inflammation, and metabolic disorders.

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