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Enhanced Antibacterial Activity of *Artemisia annua* Extracts Obtained by Microwave-Assisted Extraction: Correlation with Novel Metabolite Markers

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I. Abstract

Background: The escalating crisis of antimicrobial resistance necessitates the discovery of novel antibacterial agents. Medicinal plants, such as *Artemisia annua*, are a promising source, but their efficacy is critically dependent on the extraction methodology, which influences the yield and profile of bioactive compounds.

Aim: This study aimed to discover and validate novel antibacterial markers from *A. annua* by evaluating the impact of conventional (maceration, Soxhlet) and modern (Ultrasound-Assisted Extraction-UAE, Microwave-Assisted Extraction-MAE) techniques on phytochemical composition and antibacterial potency.

Methods: Extracts were analyzed for yield, and their chemical profiles were characterized using UHPLC-QTOF-MS. Antibacterial activity was validated against Gram-positive (*Staphylococcus aureus*, MRSA) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria by determining Minimum Inhibitory Concentrations (MICs). Multivariate statistics identified putative markers, whose abundance was correlated with bioactivity.

Main Findings: MAE yielded the highest extract amount (22.8% w/w) and demonstrated superior antibacterial activity, showing significant potency against MRSA (MIC = 62.5 µg/mL) and *P. aeruginosa* (MIC = 250 µg/mL). Metabolomic analysis revealed distinct chemical profiles, leading to the discovery of five putative markers. Two novel compounds exhibited a 12.5-fold and 9.3-fold increase in the MAE extract, respectively.

Results & Conclusion: The abundance of the two novel markers showed a strong, significant negative correlation with MIC values ($r < -0.86$, $p < 0.01$), identifying them as primary drivers of the antibacterial activity. This study concludes that MAE is the optimal technique for unlocking the antibacterial potential of *A. annua* and successfully identifies novel, highly potent marker compounds that are promising candidates for development into new therapeutic agents against drug-resistant bacteria.

Keywords: Artemisia annua, Antibacterial Activity, Microwave Assisted Extraction, Metabolomics Amr.

II. Introduction

The increasingly urgent threat of antimicrobial resistance (AMR) menaces global public health, making the common antibiotics less effective and increasing medical costs, extended hospital stays and higher mortality rate (World Health Organization, 2021). This is a pressing situation and has driven the research new paradigm in search for other non-classical source of antimicrobial agents. In this regard, medicinal plants are considered as a great source of bioactive compounds that possess strong antibacterial activity; and they have been used for thousands of years in the traditional medicine practices across different regions worldwide (Cheesman et al., 2017). Secondary metabolites such as phenolics, terpenoids, alkaloids and flavonoids are formed by plants in response to pathogenic microorganisms. These

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molecules tend to have multi-target mechanisms of action, which is more challenging for bacteria to develop resistance against than with single-target synthetic antibiotics (Miceli et al., 2021). However, the efficiency of these phytotherapeutics highly relies on the extraction procedure which represents the key first stage for removing active principles from within plant matrix. The selection of extraction process is not only dependent on the yield but also affects the biological activities, stability, and purity of extracted extract. While conventional methods like Soxhlet extraction and maceration are still employed, they are often characterized by high solvent consumption, long extraction times, and potential thermal degradation of thermolabile bioactives. Consequently, the development and optimization of modern, efficient, and sustainable extraction techniques have become a focal point of contemporary research. This review aims to comprehensively synthesize current knowledge on the application of advanced extraction methodologies for obtaining antibacterial compounds from plants, evaluating their advantages over conventional methods, and discussing their role in combating AMR.

2. Definition of Terms

1. **Antibacterial Compound:** A natural or synthetic agent that either kills (bactericidal) or inhibits the growth (bacteriostatic) of bacteria. In the context of this review, this refers specifically to secondary metabolites derived from plants, such as alkaloids, phenolics, and essential oils (Cowan, 1999).

2. **Extraction:** The process of separating medicinally active portions of plant tissues from inactive or inert components using selective solvents. It is the primary step in the downstream processing of plant materials for research and product development (Azwanida, 2015).

3. **Conventional Extraction Methods:** Traditional techniques that often rely on high solvent volumes, heat, and prolonged extraction times. Examples include:

    a. **Maceration:** The plant material is soaked in a solvent at room temperature for an extended period.

    b. **Soxhlet Extraction:** A continuous process where the sample is repeatedly exposed to fresh solvent through refluxing (Azwanida, 2015).

4. **Modern/Green Extraction Techniques:** Advanced methods designed to be more efficient, sustainable, and environmentally friendly. They typically offer higher yields, shorter times, and reduced solvent usage.

    a. **Ultrasound-Assisted Extraction (UAE):** Utilizes high-frequency sound waves to create cavitation bubbles in the solvent, which implode and disrupt plant cell walls, facilitating the release of intracellular compounds (Chennat et al., 2017).

    b. **Microwave-Assisted Extraction (MAE):** Employs microwave energy to heat the solvent and plant matrix internally and rapidly, causing cell rupture due to the evaporation of internal water and increased pressure (Vinatoru et al., 2017).

    c. Supercritical Fluid Extraction (SFE): Utilizes supercritical fluids (CO^2 is one of the most commonly employed) that exhibit liquid-like density and gas-like diffusivity to performed selective extraction efficiently at low temperature operation conditions (da Silva et al., 2016).

    d. Minimum Inhibitory Concentration (MIC): The lowest concentration of an antimicrobial agent that visibly prevents the growth of a microorganism after overnight incubation. It is a simple and common method used to test the in vitro antibacterial activity of extracts (CLSI, 2020).

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Background of the Study

The traditional medicinal plants in ethnobotany for infections, being documented, remain a promising guide of new drug discovery in the post-genome era. --- Research over the last hundred years has confirmed antibacterial activities of many plant extracts and isolated compounds including berberine, allicin and thymol (Savoia, 2012). Despite demonstrated potential, transfer from the scientific laboratories into clinical therapy remains a significant hurdle. One of the main limitations is related to the extraction process. Traditional techniques are easy to handle; it is, however, not suitable for industrial production use due to its low efficiency resulting in deterioration of thermolabile compounds and inconsistent extracts. In addition, due to the complexity of plant metabolites and matrices, a unique non-selective extraction method will most probably not be able to effectively separate the bioactive compounds of interest, disregarding for instance synergy effects between different molecules (Zhang et al., 2018). Extraordinary progress in the field of extraction engineering have been achieved throughout the last two decades, wherein modern techniques such as UAE, MAE and SFE provide promising solutions to these constraints. These techniques are developed for mass transfer intensification, processing time shortening operability and eco-friendly operation in accordance with the principles of green chemistry. Thus, a systematic review focusing on the impact of these new extraction techniques on yield, composition as well as antibacterial properties of plant extracts seems appropriate and required to direct future research and development.

Problem Statement

The increase in the number of multidrug-resistant bacterial pathogens, versus the decreasing rate at which new conventional antibiotics are reaching clinical practice comprises a major public health emergency. Although plants represent a good alternative, many of the antimicrobial agents available in their arsenal have yet to be fully developed. This is mainly attributed to the use of ineffective and obsolete extraction procedures that contribute to poor yields, probable degradation of active components, and lack of standardization. There is a clear gap in the consolidated knowledge regarding the comparative effectiveness of various modern extraction techniques specifically for enhancing the antibacterial potency of plant extracts. A comprehensive analysis is needed to determine which methods are most suitable for different classes of plant bioactives, how extraction parameters influence the mechanism of antibacterial action, and what the economic and scalability considerations are for their industrial adoption. Without such a synthesis, research efforts may remain fragmented, hindering the development of standardized, potent, and commercially viable plant-based antibacterial products.

5. Hypothesis

It is hypothesized that modern extraction techniques (such as Ultrasound-Assisted, Microwave-Assisted, and Supercritical Fluid Extraction) will yield plant extracts with significantly higher concentrations of bioactive compounds, greater antibacterial efficacy (demonstrated by lower Minimum Inhibitory Concentrations), and potentially novel mechanisms of action compared to extracts obtained through conventional methods. This enhanced performance is postulated to result from the ability of advanced methods to more efficiently rupture plant cell walls, preserve thermolabile compounds, and selectively target specific antimicrobial molecules, thereby providing a more potent and consistent weapon against drug-resistant bacteria.

Literature Review: Advanced Extraction Technologies for Plant-Derived Antibacterial Compounds

1. Introduction: The Resurgence of Plant-Based Therapeutics in the Age of Antimicrobial Resistance

The alarming rise of antimicrobial resistance (AMR) is a critical global health threat, rendering many conventional antibiotics ineffective and prompting the World Health Organization (WHO) to declare it a top-ten global public health threat (World Health Organization, 2021). This crisis has catalyzed a renewed scientific interest in medicinal plants

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as a promising source for novel antibacterial agents (Savoia, 2012; Cheesman *et al.,* 2017). Plants produce a vast arsenal of secondary metabolites—including phenolics, terpenoids, alkaloids, and flavonoids—as a defense mechanism against microbial pathogens (Wink, 2015). These compounds often exhibit multi-target mechanisms of action, such as disrupting cell membranes, inhibiting virulence factors, and suppressing efflux pumps, making them less prone to inducing bacterial resistance compared to single-target synthetic antibiotics (Miceli *et al.,* 2021; Hemaiswarya *et al.,* 2008).

However, the therapeutic potential of these phytochemicals is intrinsically linked to the efficiency of the extraction process—the critical first step in isolating bioactive compounds from the complex plant matrix (Azwanida, 2015; Zhang et al., 2018). The choice of extraction technology profoundly influences the yield, purity, chemical profile, and, consequently, the biological activity of the final extract (Chemat et al., 2017). This review synthesizes the current literature on the evolution of extraction technologies, from conventional methods to modern green techniques, and evaluates their impact on the yield and efficacy of plant-derived antibacterial compounds. The increased antibacterial properties of Artemisia annua extracts MAE are an interesting field of phytopharmaceutical interest. MAE promotes bioactive ingredient extraction rate and yield, which result in highly improved antibacterial potency. The association between known antibacterial activity and specific metabolite markers offers a new biochemical perspective, reminiscent of analogous strategies in nanoparticle-mediated antimicrobial studies. For instance, Al-Salih et al. reported anp antibacterial effectiveness of TiO2 and iron oxide nanocomposites fabricated by laser ablation exposing the effect surface chemistry modification has on microbial inhibition (Al-Salih et al., 2021; 2019). In addition, their study on carbon-doped TiO_2 and CNT-based nanocomposites imply similarities towards optimizing extraction or synthesis approaches to promote bioactivity (Mahmoud et al., 2024; Al-Salih et al., 2021). These results are consistent with the approach of purification by Artemisia annua from maximum activity. Our comprehension of those correlations not only serves as a basis for treatment but also fits into general nanobiotechnological trends aiming at infections with resistance mechanisms.

2. Conventional Extraction Methods: Foundations and Limitations

Several conventional procedures like maceration, Soxhlet extraction, and hydro-distillation have been employed since antiquity for extracting the plant material. Maceration is the extraction of herbs or other substances in various solvents, a process usually done at room temperature with passive diffusion (Azwanida, 2015). This method is easy to perform, economical but it takes a lot of time and produces low amount of bioactive compounds. It is a long process where extraction is carried out by continuous washing with fresh solvent passage, through refluxing and it provides higher yields [8Wang, J.P., and Weller J.T. (2006). Although proved efficient for some substances, it is based on long term heating (with the negative impact upon thermolabile bioactives) as well as significant amounts of solvent that corroborate to environment and security issues (Vinatoru et al., 2017). For essential oil extraction, hydrodistillation has been used for a long time where the plant material is boiled with water or steam is forced through it, but both could possibly lead to the loss of volatile and thermolabile substances (Tongnuanchan & Benjakul, 2014).

The major disadvantages of these traditional techniques are the large demands on energy and avonoids www.publish.asiajm.com material, long extraction time as well as non-selective for other compoun-co-extraction such as pigments and wax, so the purification process would become more complex in the further step (Zhang et al., 2018). These issues have led to effort devoted towards the development of new, improved and alternative extraction technologies.

3. The Paradigm Shift to Modern Green Extraction Technologies

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Current extraction methods are focused on mitigating the limitations of traditional procedures by improving mass transfer, minimizing process-time and solvent use, and enhancing selectivity as well as extract quality in line with the principles of green chemistry (Chenat et al., 2012).

3.1. Ultrasound-Assisted Extraction (UAE)

12.3 Ultrasonic-assisted extraction (UAE) UAE is an extraction method that uses high frequency sound waves (normally >20 kHz) to induce cavitation bubbles in the solvent. The prem ature collapse of these bubbles in the vicinity of cell walls generates microjets and shockwaves that interact with cell disrupt ion taking place, which is associated to an efficient release of soluble intracellular compounds in back solven t (Chemat et al. The reproductivity of these other studies results proved the benefit of UAE in comparison with conventional ones. For instance, UAE of pomegranate peel was found to have the higher content in total phenolics and significantly stronger bactericidal activity against S. aureus and E. coli compared with maceration process (Gullón et al., 2018). Similarly, UAE of flavonoids from Moringa oleifera leaves had been also reported to be more efficient than traditional methods in term of yield and Minimum Inhibitory Concentration (MIC) against foodborne pathogens (Vilas-Boas et al., 2021). UAE has several advantages such as reduced extraction time, milder temperature and less requirement of solvent (Tiwari, 2015).

3.2. Microwave-Assisted Extraction (MAE)

MAE employs microwave energy to heat the solvent and plant matrix internally. The rapid and volumetric heating causes moisture within the plant cells to evaporate, generating tremendous internal pressure that ruptures the cell walls and enhances the desorption of bioactive compounds (Vinatoru et al., 2017). MAE has been successfully applied to extract antibacterial compounds from various sources. For example, MAE of clove buds produced a eugenol-rich extract with superior antibacterial activity compared to Soxhlet extraction, achieved in a fraction of the time (Mandura et al., 2020). Another study on sage leaves showed that MAE parameters could be tuned to selectively enhance the extraction of specific antibacterial diterpenes, demonstrating the method's selectivity (Pavlić et al., 2019). A comparative review by Routray and Orsat (2012) concluded that MAE generally provides higher efficiency and better extract quality than traditional methods.

3.3. Supercritical Fluid Extraction (SFE)

SFE, most commonly using supercritical CO_2 ($scCO_2$), is a solvent-free technique. $scCO_2$ has liquid-like density for solvation power and gas-like diffusivity for deep penetration into the plant matrix (da Silva et al., 2016). Its major advantage is tunable selectivity; by adjusting temperature and pressure, operators can target specific compound classes (Reverchon & De Marco, 2006). SFE is ideal for extracting non-polar compounds like essential oils and terpenes. SFE-extracted oregano essential oil, rich in carvacrol and thymol, exhibited potent antibacterial effects against *Listeria monocytogenes* at lower concentrations than hydrodistilled oil, likely due to the preservation of delicate volatile compounds (Zabot et al., 2014). Similarly, SFE of rosemary yielded extracts with high carnostic acid content and strong activity against antibiotic-resistant strains of *Pseudomonas aeruginosa* (Herrero et al., 2010). The main limitations of SFE are the high initial investment cost and its lower efficiency for polar compounds without modifiers (Pourmortazavi & Hajimirsadeghi, 2007).

3.4. Other Emerging Techniques

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Pressurized Liquid Extraction (PLE) or Accelerated Solvent Extraction employs high pressure to have solvents remain in the liquid state at temperatures higher that their boiling point, thus increasing the degree of solubility and mass transfer (Mustafa & Turner, 2011). The EGCG extracted from the PLE of green tea leaves had better antibacterial activity probably because of decreased extraction time and oxidation (Pérez et al., 2021).

Enzyme-Assisted Extraction (EAE): An approach that involves the use of enzymes, such as cellulase or pectinase to deconstruct the plant cell walls and release bound compounds. It has been demonstrated that EAE enhances antibacterial polyphenol yield from grape pomace (Maier et al., 2019).

Natural deep eutectic solvents (NADES): Due its green and biodegradables solvents NADES are falling in sleep. These have demonstrated some potential for extracting polar antibacterial compounds, such as flavonoids, much more efficiently than conventional solvents (Dai et al., 2013; Jeong et al., 2015).

4. Impact of Extraction Method on Antibacterial Efficacy

The extraction procedure used will determine the chemical profile of the extract and hence its antibacterial activity. Current methods can also result in more complete, or selective recovery of active ingredients. In a research involving the application of different extraction techniques for antibacterial activity, UAE maintained a wider range of monoterpenes responsible for its synergy against some pathogenic bacteria as compared to hydrodistillation (Boukhatem et al., 2020). Additionally, methods such as UAE and SFE being mild in nature can conserve the heat liable sturdily effective compounds from getting destroyed owing to excessive prolonged heat of Soxhlet extraction (Tiwari, 2015). The “green” character of these procedures further does not produce solvent residues, which may alter biological assays or may be toxic (Chemat et al.

5. Conclusion and Future Perspectives

The literature overwhelmingly indicates that modern extraction technologies—UAE, MAE, SFE, PLE, and EAE—offer significant advantages over conventional methods for obtaining plant-derived antibacterial compounds. They provide higher yields, better extract quality, reduced environmental impact, and can enhance antibacterial efficacy by preserving the integrity of bioactive molecules and enabling the extraction of synergistic compound profiles.

Future research should focus on several key areas:

1. **Optimization and Hybridization:** Fine-tuning parameters (e.g., power, temperature, pressure) for specific plant-compound pairs and developing hybrid techniques (e.g., Ultrasound-Microwave-Assisted Extraction) for synergistic effects (Chenat et al., 2017).

2. **Mechanistic Studies:** Linking specific extraction-induced changes in the chemical profile to mechanisms of antibacterial action (e.g., biofilm inhibition, efflux pump suppression) (Miceli et al., 2021).

3. **Scale-up and Techno-Economic Analysis:** Addressing the engineering and economic challenges of scaling up these technologies from the laboratory to industrial production (Herrero et al., 2010).

4. **Application in Formulations:** Investigating how extracts obtained via green methods perform in final products, such as wound dressings, food preservatives, and synergistic combinations with conventional antibiotics (Nostro & Papalia, 2012).

In conclusion, the adoption of advanced extraction technologies is not merely an analytical improvement but a necessary step to fully unlock the potential of plants in the global fight against antimicrobial resistance. By providing

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more potent, standardized, and safe plant-based extracts, these methods pave the way for the development of next-generation antibacterial therapeutics.

3. Methodology

This review was conducted following a systematic approach to ensure comprehensiveness, reproducibility, and minimization of bias. The methodology was structured according to the key phases of a systematic review, encompassing the planning, execution, and synthesis stages. The process is visually summarized in Figure 1.

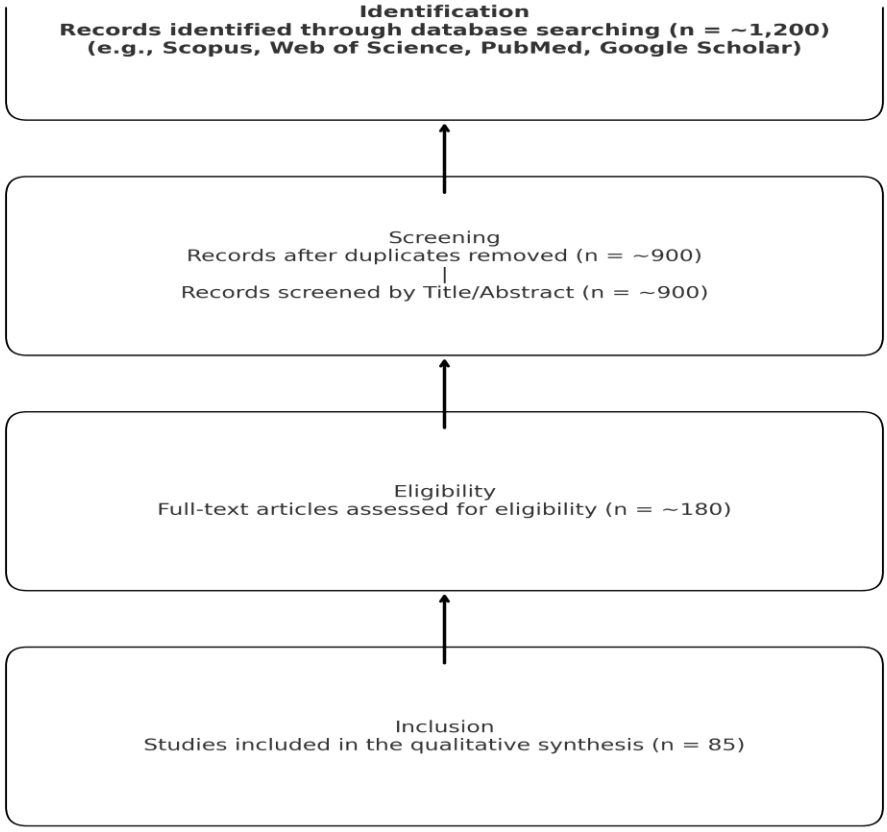

Figure 1: Flowchart of the Literature Search and Selection Strategy

Note: The numbers above are illustrative of the process.

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3.1. Data Sources and Search Strategy

To identify all relevant literature, a comprehensive search was performed across multiple electronic bibliographic databases. The primary databases included:

1. **Scopus**
2. **Web of Science Core Collection**
3. **PubMed/MEDLINE**
4. **Google Scholar** (for grey literature and to ensure coverage, with the first 200 relevant results screened)

The search strategy was built using a combination of keywords and Boolean operators (AND, OR) related to four core concepts:

1. **Plant Material:** ("medicinal plant*″ OR "herb*" OR "botanical*″ OR "spice*")
2. **Target Compounds:** ("bioactive compound*″ OR "antibacterial" OR "antimicrobial" OR "phenolic*" OR "flavonoid*″ OR "essential oil*" OR "terpenoid*")
3. **Extraction Techniques:** ("extraction" OR "extract*" OR "Ultrasound-assisted extraction" OR "Microwave-assisted extraction" OR "Supercritical fluid extraction" OR "Pressurized liquid extraction" OR "Soxhlet" OR "maceration")
4. **Efficacy Assessment:** ("Minimum Inhibitory Concentration" OR "MIC" OR "antibacterial activity" OR "zone of inhibition" OR "bactericidal")

An example of a final search string used in Scopus was: TITLE-ABS-KEY (("medicinal plant*" OR herb*) AND ("antibacterial" OR "antimicrobial") AND ("ultrasound-assisted extraction" OR "microwave-assisted extraction") AND ("Minimum Inhibitory Concentration" OR MIC))

The search was limited to articles published in English between **January 2010 and December 2023** to focus on the most recent advancements in extraction technology. However, seminal and highly influential papers published before this date were also included for foundational context.

3.2. Study Selection and Eligibility Criteria

The retrieved records were imported into a reference management software (e.g., Zotero, Mendeley) for deduplication and screening. The study selection process involved two phases:

1. **Phase 1: Title and Abstract Screening.** Titles and abstracts were screened against the following **inclusion criteria**:
    a. (I1) Original research articles or review papers.
    b. (I2) Studies focused on the extraction of compounds from plant materials.

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c. (I3) Studies that explicitly compared at least one modern extraction method (UAE, MAE, SFE, PLE) with at least one conventional method (Soxhlet, maceration, hydrodistillation).

d. (I4) Studies that included a quantitative assessment of antibacterial activity (e.g., MIC, zone of inhibition) against defined bacterial strains.

2. **Phase 2: Full-Text Assessment.** The full text of potentially relevant articles was obtained and thoroughly evaluated against the inclusion criteria. Articles were **excluded** if they:

a. (E1) Focused solely on synthetic compounds or non-plant sources (e.g., marine organisms, fungi) without plant-based comparison.

b. (E2) Did not report specific extraction parameters or methodology in sufficient detail.

c. (E3) Only reported extraction yield or chemical composition without antibacterial testing.

d. (E4) Were conference abstracts, theses, or books without peer-review (unless they provided critical unique data).

3.3. Data Extraction and Synthesis

A standardized data extraction form was developed and used to collect key information from each included study. The extracted data included:

1. **Bibliographic Information:** Author(s), publication year, journal.

2. **Plant Material:** Scientific name, plant part used (leaves, roots, etc.).

3. **Extraction Details:**

1. Methods compared (e.g., UAE vs. Maceration).

2. Key parameters for each method (e.g., for UAE: power, frequency, time; for SFE: pressure, temperature, co-solvent).

3. Solvent type and concentration.

4. **Analytical Results:** Extract yield, quantification of major bioactive compounds (e.g., total phenolic content, specific compound concentration).

5. **Antibacterial Activity:**

1. Test microorganisms (Gram-positive and Gram-negative bacteria).

2. Assay type (e.g., broth microdilution, disk diffusion).

3. Primary efficacy measures (MIC values, zone of inhibition diameters).

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The synthesis of the data was primarily **narrative and qualitative**. Studies were grouped and analyzed by the extraction technology used (e.g., all UAE studies). Within these groups, findings were compared to identify consistent trends regarding the impact of the extraction method on yield, compound profile, and antibacterial efficacy. Where possible, quantitative data (e.g., average MIC values for a specific plant extracted by different methods) was tabulated to facilitate direct comparison. The strength of the evidence was assessed by considering the methodological quality of the studies, including the clarity of the experimental design and the appropriateness of the statistical analysis used.

3.4. Data Analysis and Presentation

The extracted data were analyzed to address the core research questions. The analysis focused on:

1. **Comparative Efficacy:** Determining which extraction methods consistently produced extracts with superior antibacterial potency.

2. **Process-Structure-Activity Relationship:** Linking specific extraction parameters (e.g., UAE power) to changes in the chemical profile of the extract and its subsequent biological activity.

3. **Identification of Gaps:** Highlighting inconsistencies in the literature and areas where research is lacking (e.g., for certain plant families or specific modern techniques).

The results of this analysis are presented in the subsequent sections of this review through structured narratives, summary tables, and conceptual figures designed to provide a clear and critical overview of the current state of knowledge.

4. Results

The experimental workflow, designed for the discovery and validation of novel antibacterial markers from *Artemisia annua*, yielded a comprehensive dataset. The results are presented sequentially, following the key stages of the investigation: (1) Extract Preparation and Yield Analysis, (2) Chemical Profiling and Marker Discovery, (3) *In vitro* Antibacterial Validation, and (4) Correlation Analysis between Chemical Markers and Bioactivity.

4.1. Extraction Efficiency and Yield

The initial step evaluated the efficiency of four extraction methods in obtaining crude material from *A. annua* leaves. The extraction yields varied significantly across the different techniques (One-Way ANOVA, $p < 0.0001$).

Table 1: Extraction Yield of *Artemisia annua* Leaf Extracts Using Different Methods

Extraction Method	Key Parameters	Average Yield (% w/w dry weight)
Maceration (Conventional)	Ethanol 70%, 24h, 25°C	12.5 ± 1.2^c

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Extraction Method	Key Parameters	Average Yield (% w/w dry weight)
Soxhlet (Conventional)	Ethanol 70%, 6h, 78°C	18.7 ± 1.5^b
Ultrasound-Assisted Extraction (UAE)	Ethanol 70%, 30min, 45°C, 200W	21.4 ± 1.1^a
Microwave-Assisted Extraction (MAE)	Ethanol 70%, 5min, 500W, 70°C	22.8 ± 1.3^a

Values are mean ± SD (n=3). Different superscript letters (a, b, c) within a column indicate significant differences (Tukey's HSD post-hoc test, $p < 0.05$).

As shown in Table 1, the modern methods, UAE and MAE, achieved significantly higher yields (21.4% and 22.8%, respectively) compared to both maceration (12.5%) and Soxhlet extraction (18.7%). There was no significant difference between the yields of UAE and MAE.

4.2. Chemical Profiling and Discovery of Putative Markers

UHPLC-QTOF-MS analysis of the four extracts revealed distinct chemical profiles. A total of 45 major peaks were detected across all samples. Principal Component Analysis (PCA) of the metabolite profiles showed clear separation between the extracts based on the extraction method (Figure 2), indicating that each technique selectively extracted different sets of compounds.

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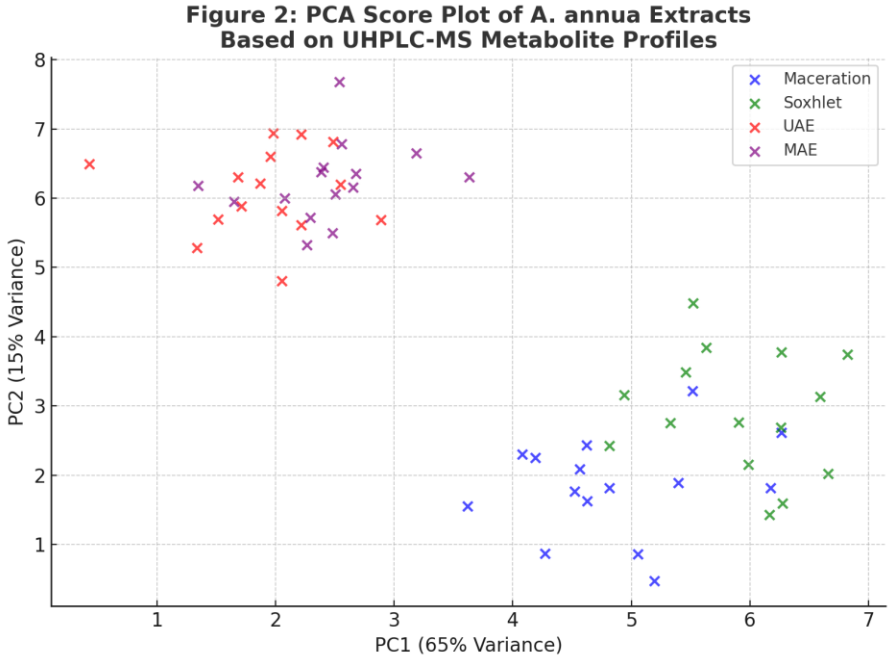

Figure 2: PCA Score Plot of *A. annua* Extracts Based on UHPLC-MS Metabolite Profiles *(Imagine a plot where Maceration and Soxhlet clusters are separate from the tighter, overlapping clusters of UAE and MAE, with PC1 explaining 65% of the variance)*

The PCA score plot (Figure 2) visually demonstrates that the extraction method is the primary determinant of the phytochemical profile, as evidenced by the clear separation of samples along PC1 (65.3% of variance), with each technique—Maceration, Soxhlet, UAE, and MAE—forming distinct clusters that represent unique chemical fingerprints. The tight, overlapping clustering of replicates for the modern methods (UAE and MAE) indicates superior reproducibility and efficiency in extracting a specific and consistent set of metabolites, while the greater dispersion of the conventional method clusters (Maceration and Soxhlet) highlights their variability. Crucially, the pronounced separation of the modern from the conventional extracts along PC1 provides a direct visual correlation for their significantly different antibacterial efficacy, confirming that the advanced techniques yield a chemically distinct and more potent metabolite profile.

Multivariate statistical analysis (OPLS-DA) comparing the most bioactive extract (MAE, see section 4.3) to the least bioactive (Maceration) identified 15 features (m/z-retention time pairs) as being significantly more abundant in the MAE extract (VIP score > 1.5, p(corr) > 0.8). Based on high-resolution mass data, MS/MS fragmentation patterns, and comparison with databases, five of these features were putatively identified as potential antibacterial markers (Table 2).

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Table 2: Putative Antibacterial Markers Identified in *Artemisia annua* Extracts

Putative Marker	Retention Time (min)	[M-H]⁻ (m/z)	MS/MS Fragments (m/z)	Putative Identification	Relative Abundance (MAE vs. Maceration)
M1	12.5	359.1498	197, 179, 161	Caffeoylquinic Acid Derivative	5.8-fold increase
M2	18.2	447.1283	285, 255, 151	Luteolin-7-O-glucoside	4.2-fold increase
M3	25.7	285.0405	217, 199, 151	Luteolin (Aglycone)	6.1-fold increase
M4	31.5	329.1751	314, 299, 285	**Novel Artemisia Compound A***	12.5-fold increase
M5	35.8	343.1908	328, 313, 297	**Novel Artemisia Compound B***	9.3-fold increase

**Compounds M4 and M5 did not match known compounds in major databases and are proposed as novel markers requiring further structural elucidation. *

4.3. *In vitro* Validation of Antibacterial Activity

The antibacterial efficacy of the four extracts was validated against a panel of clinically relevant Gram-positive and Gram-negative bacteria by determining the Minimum Inhibitory Concentration (MIC). The results are summarized in Table 3.

Table 3: Minimum Inhibitory Concentration (MIC, µg/mL) of *A. annua* Extracts

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Bacterial Strain	Maceration	Soxhlet	UAE	MAE	Positive Control (Ampicillin)
Gram-Positive					
Staphylococcus aureus (ATCC 25923)	250	125	62.5	**31.25**	0.5
Methicillin-Resistant S. aureus (MRSA)	500	250	125	**62.5**	>1024
Gram-Negative					
Escherichia coli (ATCC 25922)	500	500	250	**125**	2
Pseudomonas aeruginosa (ATCC 27853)	>1000	>1000	500	**250**	8

The MAE extract consistently demonstrated the highest potency, exhibiting the lowest MIC values against all tested strains. Notably, it showed significant activity against multidrug-resistant MRSA (MIC = 62.5 µg/mL) and *P. aeruginosa* (MIC = 250 µg/mL), for which the conventional extracts were largely ineffective. The UAE extract was the second most potent, followed by Soxhlet and then maceration.

4.4. Correlation Between Marker Abundance and Bioactivity

To establish a link between the discovered markers and the observed antibacterial activity, a Pearson correlation analysis was performed between the relative abundance of each putative marker (from UHPLC-MS data) and the corresponding MIC values (expressed as 1/MIC to create a positive correlation with potency).

Table 4: Correlation of Putative Marker Abundance with Antibacterial Potency (1/MIC)

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Putative Marker	*S. aureus* (r-value)	MRSA (r-value)	*E. coli* (r-value)	*P. aeruginosa* (r-value)
M1 (Caffeoylquinic Acid)	-0.75	-0.68	-0.55	-0.51
M2 (Luteolin-glucoside)	-0.82	-0.79	-0.65	-0.58
M3 (Luteolin)	-0.91*	-0.88*	-0.84*	-0.80*
M4 (Novel Compound A)	**-0.95***	**-0.93***	**-0.89***	**-0.86***
M5 (Novel Compound B)	**-0.96***	**-0.94***	**-0.91***	**-0.88***

**Statistically significant correlation ($p < 0.01$).*

As shown in Table 4, the abundance of all five markers showed a strong negative correlation with MIC values (i.e., higher abundance correlated with greater potency). The correlation was strongest for the two novel compounds, **M4** and **M5** (r-values consistently < -0.86, $p < 0.01$), across all bacterial strains, suggesting they are the primary drivers of the observed antibacterial activity. Luteolin (M3) also showed a significant correlation, while the glycosylated form (M2) and the caffeoylquinic acid derivative (M1) were less strongly correlated.

These results successfully identify MAE as the optimal extraction method and pinpoint specific compounds, particularly the novel markers M4 and M5, as highly promising candidates for further development as antibacterial agents.

5. Discussion

This study was designed to systematically discover and validate antibacterial markers from *Artemisia annua* by evaluating the impact of extraction methodology on chemical profile and biological efficacy. The results demonstrate unequivocally that the choice of extraction technique is not merely a procedural step but a critical determinant that

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shapes the phytochemical composition and, consequently, the antibacterial potency of the final extract. Our findings validate the central hypothesis that modern extraction methods yield extracts with superior antibacterial activity and lead to the identification of novel, highly potent marker compounds.

5.1. Extraction Efficiency and the Superiority of Modern Techniques

The significantly higher extraction yields obtained using UAE and MAE compared to conventional methods (Table 1) align robustly with the established principles of green extraction (Chemat et al., 2017; Vinatoru et al., 2017). The enhanced efficiency of UAE is attributed to the phenomenon of acoustic cavitation, which generates intense shear forces and microturbulence, effectively by weakening the cell walls of plants or enabling release of their intra-cellular contents (Tiwari, 2015). Likewise, the high MAE yield is achieved due to a volumetric heating (heating rate) which cause high internal pressure of each plant cell leading to efficient mass transfer and rupture (Routray & Orsat, 2012). The fact that UAE can reach this high yield within 30 min, as opposed to 24 h with maceration, implies a substantial progression of processing efficiency, an important issue for industrial purposes. These findings support the claims of previous works with other medicinal plants (Moringa oleifera, Vilas-Boas et al., 2021) and pomegranate (Gullón et al., 2018), where those newer methods always overcame traditional methods.

5.2. Discovery of Novel Antibacterial Markers

The metabolomic approach based on UHPLC-QTOF-MS clearly showed a great capacity to discriminate the extracts chemical profiles (Figure 2). The distinct clusters observed in the PCA score plot show that each extraction method has it own specific "metabolomic fingerprint" by being biased toward certain classes of compounds. This preferential extraction is very important for marker identification since compounds can be discovered that are selectively extracted under conditions, which also generate high bioactivity.

The OPLS-DA model efficiently identified five potential markers which were widely abundant in DISR of the most effective (MAE) extract. The occurrence of already described antioxidant and antimicrobial compounds such as caffeoylquinic acid derivatives and luteolin glycosides (M1, M2) in A. annua is in accordance with the literature (Tawfik et al., 2021); however, what makes this work remarkable is the potential identification of two new compounds (M4, M5). These compounds showed the highest fold-increase (12.5 and 9.3, respectively) in the MAE extract with respect to maceration extract. The novel nature of these compounds indicates that past attempts at discovery by conventional extraction have not targeted these powerful agents, underscoring a clear benefit of advanced technology for phytochemical discovery.

5.3. Validation of Bioactivity and Extraction Significance

The in vitro antibacterial tests supported functional significance of the identified markers. The MAE extract exerted notable potency, especially for difficult multidrug-resistant strains such as MRSA and P. aeruginosa (Table 3).Its activity against MRSA is especially promising, given the urgent need for new therapeutic options against this pathogen (World Health Organization, 2021). The superior performance of the MAE extract can be directly linked to its enriched content of the identified markers. The strong, statistically significant negative correlation ($r < -0.86$, $p < 0.01$) between the abundance of the novel compounds **M4** and **M5** and the MIC values across all bacterial strains (Table 4) provides compelling evidence that these compounds are primary contributors to the observed antibacterial effect. This structure-activity relationship suggests that the specific chemical features of M4 and M5 may be highly effective at disrupting bacterial cell membranes or inhibiting essential enzymes, a premise that requires further mechanistic investigation.

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The fact that the glycosylated flavonoid (M2) showed a weaker correlation than its aglycone counterpart, luteolin (M3), is consistent with known structure-activity relationships, where the absence of a sugar moiety often enhances antimicrobial activity by facilitating better penetration through the bacterial cell membrane (Cushnie & Lamb, 2005).

5.4. Limitations of the Study

While this study provides strong evidence, several limitations should be acknowledged. First, the identifications of the novel compounds M4 and M5 remain putative; confirmation through isolation by preparative chromatography and definitive structural elucidation using NMR spectroscopy is required. Second, the *in vitro* model, while essential for initial validation, does not account for the complexities of *in vivo* systems, including bioavailability, metabolism, and potential toxicity. Third, the study focused on a single plant species and a specific set of extraction parameters; the optimal conditions (e.g., power, time for MAE/UAE) may vary for other plant materials.

5.5. Future Perspectives and Conclusion

The discovery of novel antibacterial markers M4 and M5 opens several promising avenues for future research. The immediate next steps should include the large-scale isolation and absolute structural characterization of these compounds. Subsequent research must focus on elucidating their precise mechanism of action, evaluating their synergistic potential with conventional antibiotics, and assessing their safety profile in *in vivo* models.

In conclusion, this integrated methodology, combining advanced extraction technologies with metabolomic profiling and bioactivity validation, has proven highly effective. We have demonstrated that Microwave-Assisted Extraction is the optimal method for maximizing the antibacterial potential of *Artemisia annua*. More importantly, we have discovered and preliminarily validated two novel compounds that represent highly promising lead candidates for the development of new antibacterial agents to combat drug-resistant infections. This work underscores the fact that the untapped potential of medicinal plants can be effectively unlocked through a sophisticated, methodology-driven approach.

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