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BIOCHEMICAL CHARACTERIZATION OF SECONDARY METABOLITES IN SELECTED MEDICINAL PLANTS

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ABSTRACT

The study includes a complete biochemical analysis of the secondary metabolites present in three essential medicinal plants which possess potential health benefits. The scientists used the combination testing method which included High-Performance Liquid Chromatography with Photodiode Array detection and Gas Chromatography-Mass Spectrometry to measure the primary active compounds present in the plant materials. The chromatographic analysis detected triterpenoid compounds at significant levels which included Azadirachtin that existed in *Azadirachta indica* at 0.82% w/w and the steroidal lactone Withaferin A which appeared in *Withania somnifera* at 1.45%. The *Ocimum sanctum* plant has a high phenolic content which includes eugenol as an aromatic component that constituted 3.12% (w/w) of the analyzed extract. The research shows that the studied species produce multiple secondary metabolites which possess unique chemical characteristics that enhance their medicinal properties. The researchers studied which plants generate terpenoids and phenolic compounds through the Mevalonate and Shikimic acid biosynthesis pathways. The research shows that plants produce specific metabolites which show different distribution patterns in their leaf and root systems. The research establishes specific indicators which use defined retention times and mass-to-charge ratios as standardized testing methods for pharmaceutical quality control and development of reliable plant-based medical products.

Keywords: Secondary Metabolites, High-Performance Liquid Chromatography, Photodiode Array Detection, Gas Chromatograph-Mass Spectrometry, Mevalonate and Shikimic Acid Biosynthesis Pathways etc

INTRODUCTION

CONCEPTUAL FRAMEWORK OF SECONDARY METABOLITES

Plants create secondary metabolites as their primary chemical production process which results in a broad range of organic compounds that display diverse chemical properties. Plants create secondary metabolites which include alkaloids and flavonoids and terpenoids and phenolic chemicals as their secondary metabolites for specific plant species Saifudin et al. (2024). The chemicals establish an advanced defense system which protects against herbivores and microbial diseases and environmental stresses that include ultraviolet light and drought conditions. The pharmaceutical industry uses these metabolites as "bioactive blueprints," which form the basis for nearly 40% of all modern synthetic drugs that range from analgesics to complex anti-carcinogenic drugs. The biochemical characterization process becomes essential because their specific environmental triggers lead to their production of complex molecular structures which laboratory synthetic methods cannot duplicate.

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Transparency: The authors affirm that this manuscript presents an honest, accurate, and transparent account of the study. All essential aspects have been included, and any deviations from the original study plan have been clearly explained. The writing process strictly adhered to established ethical standards.

IMPORTANCE OF CHOSEN MEDICINAL PLANTS

The Indian subcontinent which has its own unique mega-biodiversity contains multiple medicinal plants which people have used in Ayurvedic and Unani treatment for over two thousand years. The three medicinal plants *Azadirachta indica* Neem as well as *Ocimum sanctum* Tulsi & *Withania somnifera* Ashwagandha share their unique distinct medicinal properties while also holding deep cultural value here Chaachouay and Zidane (2024). The limonoids of *Azadirachta indica* display their complex nature through their presence in Azadirachtin which functions as an insecticide and anti-inflammatory agent by blocking specific hormonal pathways in insects. The plant *Ocimum sanctum* which people call the "Queen of Herbs" contains essential oils and phenolic acids in high amounts which work together to establish its status as a strong adaptogen that helps humans manage both metabolic and psychological stress. *Withania somnifera* contains special steroidal lactones called withanolides which scientists recognize for their ability to protect brain cells and regulate immune function offering new pathways to treat neurological disorders Alam et al. (2022).

THE DISCREPANCY IN EXISTING RESEARCH ANALYSIS

Despite their historical success and widespread use these plants still need complete biochemical profiles which can meet international requirements for pharmaceutical use. Current research mainly discusses either crude extracts or basic chemical assessments because they do not provide detailed chemical profiles which advanced chromatography methods can identify Yan and Xia (2024). The different soil types and elevation levels together with the various extraction solvents lead to different metabolite outputs which may affect how well therapies work and how safe they are for patients. Numerous studies fail to prove specific metabolites exist in plants because their production originates from either the Mevalonic pathway or Shikimic acid pathway. The research uses High-Performance Liquid Chromatography HPLC and Gas Chromatography Mass Spectrometry GC-MS to create an accurate quantitative and qualitative analysis. This research aims to link traditional plant knowledge with modern evidence-based pharmacognosy through its detailed scientific study.

MATERIALS AND METHODS

PLANT MATERIAL COLLECTION AND STANDARDIZED PREPARATION

The successful outcome of phytochemical and biochemical identification depends on three essential steps which include selecting, authenticating, and preparing plant materials. The current research project involved collecting fresh leaves from *Azadirachta indica* and *Ocimum sanctum* and *Withania somnifera* roots at a certified botanical research station medicinal plant conservatory. The identification of plant specimens was confirmed by a qualified taxonomist, employing authenticated taxonomy and the preservation of voucher samples for future reference Chen et al. (2024). Immediately following harvest, the plant materials underwent post-harvest treatment; this involved thorough washing with deionized water to remove all soil particles, dust, and other external contaminants.

The researchers used shade-drying to preserve thermolabile secondary metabolites while protecting the samples from enzymatic degradation through controlled drying at 25 °C until they reached stable dry weight over a period of ten days. The researchers utilized a mechanical grinder equipped with a cooling jacket to create coarse powder from the dried plant materials. Afterward, the powdered material was passed through a 40-mesh sieve to create a consistent particle size throughout the sample. Scientists need to maintain consistent particle size throughout their experimental work because it impacts how solvents move through materials during extraction processes and affects their ability to extract substances.

SOLVENT EXTRACTION AND FRACTIONATION PROCEDURES

The researchers used a sequential solvent extraction method to extract all phytochemical components from the samples which included both polar and moderately non-polar substances. The researchers used Soxhlet extraction, applying 500 mL of HPLC-grade methanol (99.9% pure, Merck) to 50 g of dried plant material Saito and Arai (2024).

The researchers choose methanol as their main extraction solvent because it has high polarity and can dissolve various phytochemicals which include phenolics and flavonoids and alkaloids and glycosides. The researchers executed extraction operations for 24 cycles to achieve complete extraction of bioactive compounds which were present in the plant matrix. To protect heat-sensitive components from breaking down during prolonged heating, the researchers used a secondary cold maceration method. This method involved an aqueous-ethanolic solvent mixture, specifically a 70:30 volume ratio.

The researchers placed the powdered samples inside amber glass containers which they kept for 72 hours while shaking them at 150 rpm to improve solvent diffusion and extraction performance. The researchers used Whatman No. 1 filter paper to filter the extracts which resulted in the removal of all insoluble particles. The researchers concentrated the filtrates through reduced pressure operation which they performed with a Buchi R-300 rotary evaporator at 40 °C to eliminate the solvent while preserving delicate metabolites. The researchers stored the concentrated crude extracts in airtight glass vials which they kept at 4 °C until they performed chromatographic analysis.

CHROMATOGRAPHIC AND SPECTROMETRIC ANALYSIS

The researchers performed quantitative analysis on Withaferin A and Azadirachtin phytochemical markers through their work with the Waters Alliance e2695 High-Performance Liquid Chromatography system which included a 2998 Photodiode Array detector. The researchers achieved separation through their use of a SunFire C18 column which operated at 30 °C and had dimensions of 250 mm × 4.6 mm with 5 µm particle size. The mobile phase contained two solvents which included solvent A that had 0.1% orthophosphoric acid dissolved in water and solvent B that used HPLC-grade acetonitrile. The researchers used a gradient elution method, changing the concentration of solvent B from 20% to 80% over 45 minutes, while keeping a constant flow rate of 1.0 mL min^{-1} Bruce (2022).

The researchers used 20 µL as their injection volume while they set detection wavelengths to 227 nm for withanolides and 214 nm for limonoids in order to achieve their highest sensitivity. The researchers used an Agilent 7890B Gas Chromatograph system which operated with a 5977B Mass Selective Detector to perform further analysis on volatile phytochemical components. The researchers carried out separation by using an HP-5MS fused silica capillary column which had dimensions of 30 m × 0.25 mm and 0.25 µm film thickness while using helium gas as their carrier at a flow rate of 1.2 mL min^{-1}. The oven temperature program started at 60 °C and increased gradually to 280 °C at a rate of 5 °C min^{-1}. The researchers established compound identification by comparing their obtained spectra with both NIST17 and Wiley spectral libraries.

METHOD VALIDATION AND CALIBRATION

The researchers created here a more process on calibration curves for based on Withaferin A & Azadirachtin by performing more than standard sample dilutions which ranged from 1 to 100 µg mL^{-3} here in this manner. The laboratory generated these calibration curves through HPLC testing which measured peak areas in relation to specified analyte levels. The researchers applied here linear regression analysis to determine more correlation coefficients which demonstrated strong linearity through values exceeding 0.99 here Wang et al. (2023).

The researchers established LOD and LOQ limits through LOD calculation methods LOD = 3.3 π/S and LOQ calculation methods LOQ = 10 π/S. The standard deviation of the intercept represents π while the calibration curve slope represents S. The researchers tested method accuracy by adding standard solutions together with sample solutions. The results matched the findings from the relative standard deviation analysis. To ascertain accuracy, recovery studies were performed; this involved the addition of known quantities of standard compounds to previously analyzed extracts, followed by the calculation of percentage recovery Warhade et al. (2022). These calculations confirmed the method's efficacy in quantifying phytochemical content.

RESULTS

QUANTITATIVE ANALYSIS OF EXTRACTION YIELDS AND PHYTOCHEMICAL RECOVERY

The initial stage of the results required researchers to conduct gravimetric measurements of crude extracts which were obtained from three chosen medicinal plants to assess the effectiveness of their extraction methods. Among the two extraction methods used, methanol-based Soxhlet extraction consistently produced higher yields compared with the aqueous–ethanolic maceration technique. The Soxhlet system achieves this effect because it maintains continuous solvent flow while operating at high temperatures which speed up the process of phytochemical extraction from plant materials Direito et al. (2019). The methanolic leaf extract of *Azadirachta indica* produced a yield of 14.8% (w/w) which was calculated from its original dry mass. The Soxhlet extraction process of *Withania somnifera* root powder resulted in 11.2% (w/w) crude extract yield. The lowest extraction yield in *Ocimum sanctum*-based situation occurred during aqueous–ethanolic maceration which resulted in 7.5% (w/w) extraction yield here. The presence of volatile aromatic compounds in the sample results in lower extraction yield because these compounds evaporate during the solvent concentration process.

HPLC FINGERPRINTING AND RETENTION TIME VALIDATION

The analysis conducted through High-Performance Liquid Chromatography created unique chromatographic patterns which enabled scientists to identify bioactive compounds based on their retention times when compared to certified reference materials Abdelhakim et al. (2021). *Withania somnifera* showed a main peak which scientists resolved at a retention time (Rt) of 12.48 minutes because it contained the steroidal lactone Withaferin A. The peak exhibited perfect symmetry through its 1.05 factor which showed the column achieved high efficiency while maintaining peak integrity. The main limonoid compound Azadirachtin A of *Azadirachta indica* reached its maximum concentration at a retention time of 18.22 minutes. Nimbin and Salannin appeared as additional secondary metabolites which scientists detected at retention times of 24.15 minutes and 29.50 minutes, respectively. The chromatographic profile of *Ocimum sanctum* revealed two major phenolic constituents, with Eugenol appearing at 15.36 minutes and Rosmarinic acid detected earlier at 8.12 minutes. The C18 column separation system achieved separation efficiency which enabled scientists to separate compounds that exhibited different polarity characteristics. The chromatographic method analysis

demonstrated consistent retention times and peak areas which showed statistical reliability through relative standard deviation values that remained below 2% and showed reproducible results at statistically significant levels ($p < 0.05$) in the chromatographic method analysis.

GC–MS CHARACTERIZATION OF VOLATILE METABOLITES

The total ion chromatogram obtained for *Ocimum sanctum* displayed more than forty-five identifiable peaks which indicated that the sample contained multiple segregated chemical constituents. Eugenol emerged as the primary compound among all these substances because it contributed approximately 62.4% of the entire peak area Zulfiqar and Ashraf (2021). The mass spectrum of this compound displayed a molecular ion at m/z 164 and a base fragment at m/z 149 which verified its structure as an allylphenol. The researchers discovered several volatile sulfur-containing compounds and sesquiterpenes in *Azadirachta indica* which produced the plant's unique scent and biological properties. The researchers achieved accurate compound identification through spectral comparisons with the NIST database which produced match factors above 92%. The results from repeat GC-MS analyses demonstrated consistent peak intensity and retention index measurements while statistical analysis proved that metabolite patterns showed both reproducibility and significant results ($p < 0.05$) Wani et al. (2023).

QUANTITATIVE DISTRIBUTION OF BIOACTIVE METABOLITES

The study used authentic reference compounds to create external standard calibration curves which enabled researchers to measure major phytochemical constituents through quantitative analysis. The researchers established metabolite concentrations in plant extracts by plotting peak area measurements for each analyte against their corresponding known concentration values. The *Withania somnifera* root extract contained Withaferin A at 1.45% (w/w) which contributed to a total withanolide content of approximately 3.8% (w/w), demonstrating the plant material's high pharmacological value Tariq et al. (2023). The comparative statistical analysis showed that plant species here to show and exhibited distinct differences in their metabolite concentrations ($p < 0.05$), which mostly here indicates that both biosynthetic pathways & plant physiology determine the accumulation of metabolites.

Plant Species	Analyzed Plant Part	Major Metabolite	Retention Time (Rt)	Concentration (% w/w)
Azadirachta indica	Leaf	Azadirachtin A	18.22 min	0.82
Azadirachta indica	Leaf	Nimbin	24.15 min	0.45
Ocimum sanctum	Leaf	Eugenol	15.36 min	3.12
Ocimum sanctum	Leaf	Rosmarinic Acid	8.12 min	1.24
Withania somnifera	Root	Withaferin A	12.48 min	1.45
Withania somnifera	Root	Withanolide D	16.75 min	0.98

STABILITY AND PURITY ASSESSMENT

The stability assessment here in this nature showed that the metabolites stayed chemically stable in the methanolic extract in this process when stored in the refrigerator for thirty days approx. Repeated use of HPLC injections showed that the peak area measurements changed by less than 2%, this mainly indicating that the samples didn't break down much during the testing period here Liang et al. (2004).

The Photodiode Array detection method proved that all chromatographic peaks maintained identical spectral properties which showed that all detected metabolites existed without any co-eluting contaminants. The statistical evaluation of repeated measurements demonstrated that no significant storage-related degradation occurred during storage ($p < 0.05$) which established the extraction and analytical methods used in this study as dependable and strong.

DISCUSSIONS

THE LOGIC OF BIOSYNTHESIS AND THE DISTRIBUTION OF PATHWAYS

The Mevalonate (MVA) and Shikimic acid pathways do metabolic steering which leads to the discovery of biochemical profiles that emerged in the research results. The MVA pathway converts acetyl-CoA into isopentenyl pyrophosphate which produces the high triterpenoid levels that occur in *Azadirachta indica* and the steroidal lactones that exist in *Withania somnifera* Bhutani (2000). The system generates high triterpenoid concentrations. The pathway requires additional energy which explains why specific metabolites tend to emerge during specific environmental conditions. The Shikimic acid pathway produces phenolic compounds in *Ocimum sanctum* because it converts carbohydrate precursors into aromatic amino acids like phenylalanine. *Withania* and *Azadirachta* allocate resources toward complex, multi-ringed structures that act as targeted deterrents against specialized herbivores and pathogens. *Ocimum* chooses to spend its resources on phenylpropanoids because they help protect its plants through

antioxidant defense together with their ability to transmit signals. The biochemical evidence supports the idea that this process functions as an evolutionary trade-off made by organisms Kokate et al. (2005).

PARTICULARLY TISSUE-SPECIFIC DISTRIBUTION AND BIOLOGICAL APPLICATIONS

The biological needs for protection and storage capacity of plants decide which metabolites will be found in their various body parts. For example, Neem and Tulsi both produce their leaves from their respective leaves while Ashwagandha derives its leaves directly from its roots. The leaves of *Azadirachta indica* function as the main photosynthesis system of the plant because they contain the highest risk of damage from leaf diseases and insect attacks. The high limonoid content in the plant leaves provides immediate protection through chemical defense mechanisms. The *Withania somnifera* roots contain a high level of withanolides which demonstrates that the plant uses "storage-defense" mechanisms Sridharan et al. (2016). The plant uses its root system as its primary method for staying alive because it uses bitter steroidal lactones to create underground protection against both microbial rot and herbivore attacks. The metabolic cost of synthesis achieves equilibrium through compartmentalization which delivers maximum protective benefits according to a concept that plant biology understands through its optimal defense theory.

SAR, WHICH STANDS FOR STRUCTURAL ACTIVITY RELATIONSHIPS, AND PHARMACOLOGY

The therapeutic effects of these metabolites depend on their Structural Activity Relationships which describe their metabolites. The bioactivity of withaferin A depends on the unsaturated lactone ring and the epoxide function which exists in the A/B ring system because these two elements both exist. The molecule uses its structural patterns to form covalent bonds with specific protein thiols which results in proteasome inhibition and death activation of abnormal cells Hussain et al. (2015). The complex oxygenation framework of Azadirachtin functions as a powerful antagonist because it shares structural similarities with ecdysone which serves as an insect growth hormone. The hydroxyl groups on the aromatic rings of rosmarinic acid and eugenol present in *Ocimum sanctum* provide essential electron-donating capability which enables the neutralization of reactive oxygen species (ROS). The study established through its detailed characterization that the compounds' molecular geometry exists in an ideal state for their specific pharmacological targets.

AN EXAMINATION OF COMPARATIVE LITERATURE WITH CONTEMPORARY EXPRESSIONS

The research results here overly match the standard chemical profiles provided by the Indian Pharmacopoeia process. The use of HPLC-PDA and GC-MS instruments enabled us to achieve more accurate quantitative results than what previous studies had reported. The research conducted by Wagner and his team in 1996 discovered that Ashwagandha contains withanolides. Our research demonstrates a different understanding of the Withaferin A to Withanolide D ratio which functions as a key element in establishing neuroprotective product standards. Our GC-MS profiling reveals neem's volatile sulfur compounds which researchers have traditionally overlooked while explaining their interaction with limonoids Li et al. (2020). The study aims to create complete dataset which supports the evolution of traditional medicinal plants into recognized pharmaceutical ingredients for global distribution. The research establishes a direct link between basic phytochemical analysis and detailed biochemical study through its findings.

CONCLUSION

The study conducted here a complete biochemical assessment of *Azadirachta indica* & *Ocimum sanctum* as well as *Withania somnifera*. The traditional plant knowledge established a strong analytical connection with the contemporary practices used in drug development research. The research team applied High-Performance Liquid Chromatography (HPLC) and Gas Chromatography–Mass Spectrometry (GC–MS) as advanced analytical methods to create complete phytochemical profiles of the medicinal plants under investigation. The research successfully identified crucial biological markers which included Withaferin A and Azadirachtin and Eugenol. The scientific testing of these markers showed accurate results which produced reliable outcomes. The research demonstrates that the plants' healing properties arise from a combination of secondary metabolites which function together as a group. The group of metabolites contains steroidal lactones and phenolic chemicals together with terpenoids. The organism maintains strict metabolic control over the processes which produce these specific metabolites. The plant defense chemical pathways are structured around both the Mevalonate and Shikimic acid metabolic pathways.

The study results demonstrate that fundamental biological laws control how metabolites move through different parts of plant structures. The leaf samples from *Azadirachta indica* and *Ocimum sanctum* displayed higher concentrations of protective phytochemicals which they used to defend themselves against herbivores and harmful microorganisms and environmental threats. The *Withania somnifera* roots collected large quantities of withanolides which demonstrated how the plant evolved to preserve essential bioactive compounds within its underground structures that support its existence and development. The research study analysis of structural activity relationship confirms that molecular structure must remain intact during extraction and analysis because the bioactive properties of compounds depend on their precise molecular structure and functional group distribution.

The research investigates the increasing requirement for scientific guidelines which should govern herbal medicine practices. The research established specific retention times and concentration limits that analytical labs must follow to develop a reliable method for assessing the phytochemical quality of pharmaceutical products. This standardization process sets important benchmarks, ensuring product safety, consistent therapeutic effects, and easier access to herbal medicine worldwide. Further studies need to investigate the combination effects of various phytochemical components which produce their medicinal benefits.

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