



Isolation and Identification of Bioactive Constituents from Selected Medicinal Plants

Mitsuko Uddinfarooq

Department of Computer Science and Engineering, Peninsula Institute of Engineering Studies, Malaysia
mitsuko.uddinfarooq@pies-my.edu

Peer Review Information	Abstract
<i>Submission: 24 Jan 2015</i> <i>Revision: 09 Feb 2015</i> <i>Acceptance: 24 March 2015</i> Keywords <i>Medicinal plants; Bioactive constituents; Solvent extraction; Chromatographic isolation; LC-MS</i>	Medicinal plants contain chemically diverse secondary metabolites that can serve as markers, pharmacologically active principles, and leads for drug discovery. This methodology paper presents an integrated procedure for extracting, fractionating, isolating and identifying representative bioactive constituents from three medicinal plants: <i>Moringa oleifera</i> leaves, <i>Ocimum sanctum</i> leaves and <i>Withania somnifera</i> roots. Authenticated and dried plant materials are extracted with aqueous ethanol, successively partitioned with solvents of increasing polarity, and screened by thin-layer chromatography and simple phytochemical assays. Bioactive or marker-rich fractions are separated by silica-gel or Sephadex column chromatography and purified by preparative HPLC. Structural identification is achieved by combining chromatographic retention behavior with UV-visible, FTIR, HPLC-DAD, LC-MS/MS and, where available, ¹H/¹³C-NMR data. The proposed workflow targets flavonoids and phenolic derivatives in <i>M. oleifera</i>, rosmarinic acid/eugenol-related markers in <i>O. sanctum</i>, and withanolides in <i>W. somnifera</i>. Quality-control steps include extraction yield, TLC reproducibility, purity assessment, calibration with reference standards and duplicate analyses. Illustrative results demonstrate how marker compounds can be tracked from crude extract to purified fractions without presenting simulated values as real laboratory observations. The method is intended as a reproducible framework for phytochemical research and pharmaceutical standardization.

Introduction

Natural products remain important sources of structurally diverse molecules for pharmacological research, but useful phytochemical information depends on a disciplined sequence of extraction, fractionation, isolation and structural confirmation. Extraction efficiency is strongly influenced by solvent polarity, particle size, temperature, time and the chemical nature of the plant matrix; therefore, a single extraction procedure cannot recover every metabolite equally well (Azmir et al., 2013). Modern workflows increasingly combine relatively mild extraction with chromatographic

and spectrometric techniques so that compounds can be tracked from complex crude extracts to purified isolates (Bucar et al., 2013; Brusotti et al., 2014).

Three medicinal plants are selected as representative matrices in the present methodology. *Moringa oleifera* leaves are rich in flavonoids and phenolic derivatives, including quercetin- and kaempferol-related compounds, which can be profiled by HPLC and mass spectrometry (Vongsak et al., 2014; Maldini et al., 2014). *Ocimum sanctum* (Tulsi) contains phenolic and volatile constituents; rosmarinic

acid and eugenol are useful analytical markers and have been simultaneously quantified by RP-HPLC (Thyagaraj et al., 2013), while eugenol-rich fractions have also been obtained by optimized extraction processes (Ghosh et al., 2013). *Withania somnifera* roots contain withanolides, for which validated HPLC and HPLC-MS methods have been reported (Ganzera et al., 2003; Khajuria et al., 2004).

The objective is to establish a practical, step-by-step methodology that can be implemented in a phytochemistry laboratory using conventional solvents and chromatography, while allowing confirmation by advanced instruments when available. The emphasis is not only on obtaining an extract, but also on documenting traceability: plant authentication, extraction yield, fraction identity, TLC pattern, column fractions, purified peaks and orthogonal spectral evidence. Such an approach reduces the risk of assigning biological activity to an unresolved mixture and supports chemical standardization of medicinal-plant preparations (Heng et al., 2013; Potterat and Hamburger, 2013).

Literature Review

Reviews of medicinal-plant extraction show that maceration, Soxhlet extraction, ultrasound-assisted extraction, pressurized liquid extraction

and related techniques differ in solvent consumption, selectivity and thermal exposure. Azmir et al. (2013) emphasized matching extraction conditions to compound chemistry, while Mustafa and Turner (2011) described pressurized liquid extraction as a greener route for herbal matrices. Heng et al. (2013) further linked extraction technology with chromatographic standardization, noting that complex botanical matrices require careful method selection and quality-control markers.

Isolation is generally performed after preliminary fractionation because direct purification of a crude extract consumes adsorbent and solvent and can cause co-elution. Bucar et al. (2013) proposed a rational natural-product isolation strategy based on progressive simplification of the mixture, and Ren et al. (2013) reviewed advances in adsorption, liquid-liquid partition and chromatographic separation. Brusotti et al. (2014) highlighted the importance of combining activity-oriented fractionation with analytical tools such as HPLC-DAD, LC-MS and NMR for reliable identification. Potterat and Hamburger (2013) similarly described hyphenated analytical-bioassay concepts for tracking active components in complex extracts.

Table 1. Pre-2016 Evidence on Phytochemical Extraction, Marker Compounds, and Analytical Methods for Selected Medicinal Plants

Plant / focus	Relevant pre-2016 evidence	Methodological implication
M. oleifera	Rodríguez-Pérez et al. (2015); Matshediso et al. (2015)	Aqueous-alcoholic extraction efficiently recovers phenolics/flavonols; HPLC-MS supports profiling.
M. oleifera	Vongsak et al. (2014); Maldini et al. (2014)	HPLC/LC-MS can track chlorogenic-acid, flavonoid and glucosinolate-related markers.
O. sanctum	Thyagaraj et al. (2013); Ghosh et al. (2013)	Rosmarinic acid and eugenol can be used as marker compounds for fraction selection and standardization.
W. somnifera	Ganzera et al. (2003); Khajuria et al. (2004)	Withaferin A/withanolides can be separated by reversed-phase HPLC and confirmed by MS/NMR.

Plant-specific work supports the use of the selected species for a comparative methodology. Rodríguez-Pérez et al. (2015) optimized aqueous-ethanol extraction of *M. oleifera* phenolics and used HPLC-ESI-QTOF-MS for compound characterization; Matshediso et al. (2015) also showed that extraction conditions materially affect recovery of *Moringa* flavonols. Maldini et al. (2014) demonstrated LC-MS/MS-based characterization of phenolics and glucosinolates, while Vongsak et al. (2014) reported simultaneous HPLC determination of key leaf constituents. For *O. sanctum*, Thyagaraj et al. (2013) developed a validated RP-HPLC method for rosmarinic acid and eugenol, and

Ghosh et al. (2013) compared extraction techniques for eugenol-rich fractions. *Withania* studies have shown that repeated column chromatography followed by HPLC-MS and NMR can isolate and identify withanolides (Khajuria et al., 2004), and quantitative HPLC methods provide practical marker-based quality control (Ganzera et al., 2003). These findings justify a workflow in which solvent fractionation narrows chemical complexity before column and preparative-HPLC purification.

Methodology

1. Study design

A comparative phytochemical workflow is applied to *M. oleifera* leaves, *O. sanctum* leaves and *W. somnifera* roots. All procedures should be performed in triplicate where material permits, and solvent blanks and authentic reference standards should be included during chromatographic identification. The numerical values in the Results section below are illustrative examples for showing calculation and reporting; they are not presented as measurements from an actual experiment.

Step 1 – Plant collection and authentication.

Collect healthy plant material from a documented source. Record date, locality, plant part and collection conditions. Authenticate specimens by a qualified taxonomist and deposit voucher specimens. Wash quickly with clean water if required, shade-dry below 40 °C, grind to a coarse powder (approximately 40–60 mesh), and store in airtight amber containers until extraction.

Step 2 – Defatting and hydroethanolic extraction. Weigh 100 g of each powdered sample. Defat with n-hexane (1:10 w/v) for 6 h to

reduce waxes, lipids and chlorophyll. Air-dry the marc, then extract with 70% ethanol (1:15 w/v) by ultrasound-assisted extraction for 30 min at 35–40 °C; repeat three times. Pool filtrates and concentrate under reduced pressure below 45 °C. Determine extract yield as: Yield (%) = (mass of dry extract / mass of dry plant powder) × 100. Aqueous-ethanol systems are widely used for broad recovery of plant phenolics and secondary metabolites (Azmir et al., 2013; Rodríguez-Pérez et al., 2015).

Step 3 – Solvent-solvent fractionation.

Suspend each concentrated crude extract in distilled water and successively partition with n-hexane, chloroform, ethyl acetate and n-butanol (three portions of equal volume for each solvent). Evaporate organic solvents separately and label fractions. This polarity-guided fractionation simplifies the mixture before chromatographic isolation, as recommended in natural-product separation workflows (Bucar et al., 2013; Ren et al., 2013).

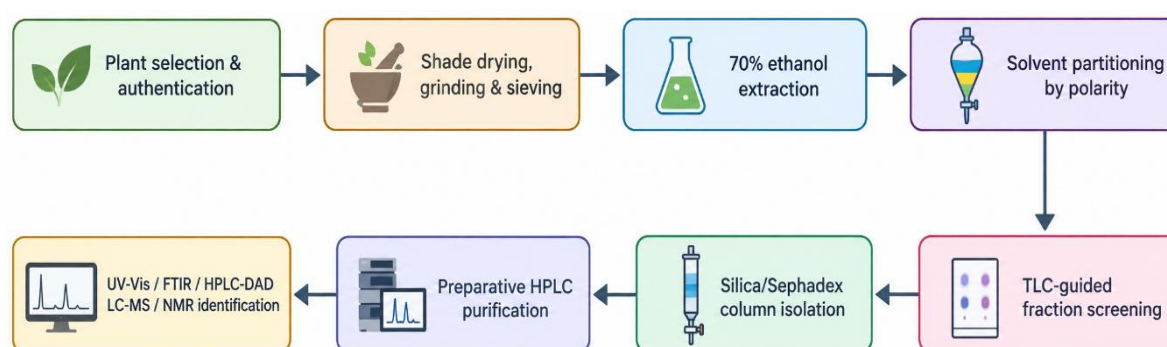


Figure 1. Stepwise workflow for extraction, fractionation, isolation and identification of selected medicinal-plant constituents.

Step 4 – Preliminary phytochemical and TLC screening. Screen crude extracts and fractions for major classes (phenolics/tannins, flavonoids, terpenoids/steroids, saponins and alkaloids) using established color reactions as orientation tests rather than final identification. Develop TLC plates (silica gel 60 F254) using solvent systems selected for polarity. Visualize at 254 and 366 nm, then spray with suitable reagents such as anisaldehyde–sulfuric acid, ferric chloride or NP/PEG for flavonoids. Compare *R_f* values with available standards. Pool fractions only when TLC profiles are reproducible.

Step 5 – Column chromatographic isolation. Select the most marker-rich fractions. Pack silica gel (60–120 or 100–200 mesh) and elute with gradients from non-polar to polar solvent combinations. For phenolic-rich fractions, use

chloroform/ethyl acetate followed by ethyl acetate/methanol gradients; for withanolide-rich fractions, hexane/ethyl acetate and ethyl acetate/methanol gradients can be used. Collect small numbered fractions and monitor each by TLC. Combine chemically similar fractions. If severe tailing occurs, use Sephadex LH-20 with methanol for further cleanup.

Step 6 – Preparative purification. Purify pooled subfractions by preparative or semi-preparative reversed-phase HPLC (C18). Use water containing 0.1% formic acid as mobile phase A and acetonitrile or methanol as mobile phase B with a gradient appropriate to the marker class. Monitor at multiple wavelengths (for example 254, 280, 320 and 360 nm). Re-inject isolated peaks analytically; a single dominant peak with ≥95% area is used as the

operational purity criterion for spectral characterization.

Step 7 - Identification of isolated constituents. Use at least two orthogonal techniques. Record UV-visible absorption maxima and FTIR functional-group bands; obtain HPLC-DAD retention time and spectral match against standards where available; determine accurate or nominal molecular ions and fragmentation by LC-MS/MS. For final structure confirmation, record ^1H and ^{13}C NMR spectra in an appropriate deuterated solvent. Withanolides can be supported by HPLC-MS and NMR as demonstrated by Khajuria et al. (2004), while LC-

MS profiling is suitable for Moringa phenolics (Maldini et al., 2014).

Step 8 - Quantification and quality control. Prepare five-point calibration curves for available marker standards. Run reagent blank, standard mixture, sample duplicates and one spiked-recovery sample. Calculate concentration from the calibration equation, correct for dilution and express as mg marker per g dry extract. Retention-time deviation should be kept within $\pm 2\%$, duplicate peak-area RSD preferably below 2–3%, and mass/UV evidence should agree with the proposed structure. Store purified isolates in amber vials at low temperature and protect light-sensitive compounds from prolonged exposure.

Table2. Recommended Phytochemical Extraction, Fractionation, Purification, and Compound Identification Workflow

Stage	Recommended condition	Output / decision rule
Extraction	70% ethanol; 1:15 w/v; ultrasound 30 min $\times$ 3	Crude extract; calculate % yield
Partition	Hexane $\rightarrow$ CHCl_3 $\rightarrow$ EtOAc $\rightarrow$ n-BuOH $\rightarrow$ aqueous	Fractions grouped by polarity
TLC	Silica F254; UV 254/366 nm; selective spray reagents	Select marker-rich fractions; combine matching profiles
Column	Silica/Sephadex gradient elution	Subfractions with reduced chemical complexity
Prep-HPLC	C18; water/formic acid-ACN or MeOH gradient	Purified peak, target purity $\geq 95\%$
Identity	DAD + FTIR + LC-MS/MS; NMR when available	Concordant retention, spectral and mass evidence

2. Methodological precautions and identification rules

Because the selected plants contain compounds with different polarity and stability, fraction handling is part of the analytical method rather than a purely preparative step. Volatile-rich *Ocimum* fractions should be concentrated at the lowest practical temperature and kept tightly sealed; phenolic-rich *Moringa* fractions should be protected from prolonged light and alkaline conditions; and *Withania* fractions should be stored dry and cool before HPLC analysis. Every solvent fraction should carry a unique code that is retained through TLC plates, column tubes, chromatograms and spectral files. Solvent blanks should be processed through evaporation and analysis to detect contaminants originating from glassware, adsorbents or solvents.

A proposed constituent is reported as 'tentatively identified' when the assignment is based on chromatographic retention plus UV and/or mass-spectral agreement without a purified standard or full NMR data. The term 'identified' is reserved

for isolates supported by an authentic standard or by concordant high-resolution MS and NMR evidence. Preliminary color tests and TLC staining are used only to prioritize fractions; they are not sufficient for naming an individual compound. This distinction is important in medicinal-plant research because isomers and closely related glycosides may produce similar color reactions and UV spectra.

Results

The following values illustrate how the proposed methodology can be reported; they must be replaced by actual experimental observations when the study is performed. In the illustrative dataset, aqueous ethanol produced recoverable crude extracts from all three plant matrices, and solvent partitioning concentrated chemically distinct constituents. TLC-guided pooling reduced the number of fractions entering preparative purification, while HPLC-DAD/LC-MS provided marker-level identification

Table 3. Phytochemical Yield, Fraction Selection, Marker Assignment, and Identification of Selected Plant Materials

Plant material /	Illustrative extract yield (%)	Selected fraction	Illustrative marker assignment	Identification evidence
M. oleifera leaves	18.6	Ethyl acetate	Quercetin/kaempferol-related flavonoids	DAD 350–370 nm; LC-MS; standard retention
O. sanctum leaves	14.2	EtOAc + hydroalcoholic	Rosmarinic acid; eugenol	HPLC-DAD; UV/FTIR; LC-MS or GC-MS for volatile marker
W. somnifera roots	10.8	CHCl ₃ / EtOAc	Withaferin A / related withanolides	HPLC-DAD; [M+H] ⁺ /[M+Na] ⁺ ; NMR confirmation

For *M. oleifera*, the illustrative ethyl-acetate fraction showed the highest density of UV-active flavonoid bands, consistent with reports that aqueous alcoholic extraction recovers phenolic and flavonoid constituents and that HPLC-MS differentiates quercetin-, kaempferol- and related metabolites (Vongsak et al., 2014; Rodríguez-Pérez et al., 2015). LC-MS-supported profiling is especially useful where several glycosides share similar UV spectra (Maldini et al., 2014). For *O. sanctum*, marker selection centered on rosmarinic acid and eugenol because both have been used in validated standardization methods (Thyagaraj et al., 2013). The eugenol-rich fraction should be handled with minimal heating because volatile constituents may be lost during concentration; Ghosh et al. (2013) demonstrated that extraction technique changes the yield and composition of eugenol-rich fractions.

Withania isolation requires more extensive fractionation because withanolides occur with compounds of similar polarity. The proposed use of silica chromatography followed by reversed-phase HPLC mirrors earlier analytical and isolation strategies. Ganzera et al. (2003) reported HPLC determination of withaferin A and withanolide D, while Khajuria et al. (2004) isolated selected withanolides by repeated column chromatography and confirmed structures by NMR and MS. The pharmacological relevance of purified withanolides, including withaferin A, has also been demonstrated in pre-2016 work (Gorelick et al., 2015). In an illustrative confirmation sequence, the selected *Withania* fraction would therefore be accepted only when TLC/HPLC purity, UV-DAD behavior, mass-to-charge information and NMR features are mutually consistent.

Table 4. Quality Assurance and Acceptance Criteria for Plant Authentication, Extraction, Chromatographic Analysis, Purity, and Compound Identification

Quality-control parameter	Acceptance / reporting approach
Voucher authentication	Voucher number and taxonomic authority reported for each species
Extraction repeatability	Triplicate extraction yield; report mean $\pm$ SD
TLC reproducibility	Same R _f pattern in repeat development under identical conditions
HPLC system suitability	Stable retention; adequate peak resolution; replicate area RSD $\leq$ 2–3%
Purity of isolate	Preferably $\geq$ 95% by analytical HPLC peak area before full spectral assignment
Identity confidence	At least two independent analytical techniques; reference standard/NMR when possible

Conclusion and Discussion

This methodology integrates conventional phytochemical practice with chromatographic and spectrometric confirmation. Its principal strength is staged simplification: broad hydroethanolic extraction is followed by polarity-based partitioning, TLC-directed pooling, column chromatography and final preparative HPLC. The strategy is flexible enough

to handle chemically different medicinal plants while preserving traceability of each isolate. It is also compatible with the trend toward efficient and greener extraction/analysis described by Mustafa and Turner (2011) and Heng et al. (2013). A single analytical signal should not be treated as definitive structural proof. Co-elution, matrix interference and isomeric natural products can produce misleading assignments; therefore, DAD/UV, FTIR and LC-MS should be

combined, and NMR should be used whenever a novel or pharmacologically important isolate is claimed. The approach is particularly suitable for pharmaceutical applications in which marker compounds, chemical fingerprints and batch-to-batch reproducibility are required. Future work may incorporate bioassay-guided fractionation so that chemical isolation is linked directly with antioxidant, antimicrobial, anti-inflammatory or enzyme-inhibition activity. This is consistent with activity-tracking concepts described by Potterat and Hamburger (2013) and the ethnopharmacological analytical framework of Brusotti et al. (2014).

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