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EXTRACTION AND PHYTOCHEMICAL ANALYSIS OF SECONDARY METABOLITES FROM *ACHYRANTHES ASPERA LINN*

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Abstract: Background: Apamarga (*Achyranthes aspera Linn*. (Family *Amaranthaceae*) is a common medicinal herb with ancient roots in Ayurvedic, Unani and Siddha systems of healing. It is used in tropical and subtropical regions for the treatment of inflammation, respiratory tract disease, gastrointestinal disorders, dermatologic conditions, kidney disease and as an antidote to snake envenomation. Despite this high ethnopharmacological recognition, a potentialwide comparative phytochemical survey of five different plant organs — roots, stems, leaves flowers and seeds using contemporary analytical methodology has not been carried out previously. **Objective**: To sequentially extract, isolate and characterize the secondary metabolite profiles of each major organ/part of this plant such as roots, bark, fruits and seed using polarity-gradient solvent extraction followed by qualitative/quantitative phytochemical screening (phytochemicals including alkaloids–the most dominant), chromatographic isolation in crude form and spectroscopic identification. **Materials and methods:** Root, stem, leaf, flower, and seed shade-dried plant materials were successively extracted using petroleum ether, chloroform, ethyl acetate, methanol and water. By using conventional methods such as maceration and Soxhlet extraction alongside green solvents, including ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). Standard qualitative phytochemical screening. Major constituents were isolated by silica gel column chromatography and their structures elucidated using UV, FTIR, 1H/13C NMR and mass spectrometry. **Results:** MAE gave the highest extract percentages for all studied plant parts (24.3–30.1% w/w for methanol), which were significantly higher than those obtained by maceration (17.1–23.0%). The column chromatography afforded fourteen characterized compounds which were identified as β-sitosterol, chlorogenic acid, betaine, achyranthine, ecdysterone, ecdysone, lupeol, oleanolic acid, rutin and quercetin and caffeic acid and stigmasterol. **Conclusions:** A. aspera has chemically diverse and part-specific secondary metabolite profile that further validates its traditional uses inpharmacotherapy. Plant Parts: Leaves are best for phenolic and flavonoid extraction; roots and seeds are optimal for alkaloids, ecdysteroids, and saponins. However, MAE is advised to be the extraction technique of choice for high yield low solvent consumption. The isolated compounds and quantitative result sets lay the scientific groundwork for quality control of herbal products while providing insight for future bioassay-directed pharmacological studies.

Keywords: *Achyranthes aspera*; Apamarga; phytochemical screening; sequential extraction; column chromatography; GC-MS; alkaloids; ecdysteroids; saponin s'; flavonoids terpenoids medicinal plants natural products.

Introduction: Medicinal plants are the oldest and most commonly used pharmaceutical resource known to man. Few things in human history have proven more tenacious than the use of plant-derived preparations for the purposes of health care, such practices documented across civilization from ancient India and into the Americas long before synthetic chemistry put forward its first drug lead. Today, even with the global domination of modern biomedicine, over 80% of the populations in developing nations continue to use exclusively plant-derived traditional medicine as their primary healthcare[1]. This fact, in the face of the worrying

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increase of failure or resistance to antievolutionary by microorganisms and also, chronic non-communicable diseases resistant to available therapies, and the incurable de novo costs on synthetic patterns for drugs development have prompted a new rapture in natural product perspectives.

Achyranthes aspera Linn. (family Amaranthaceae) holds a position of singular importance in this arena. Popularly referred to as Apamarga (literally 'that which removes disease') in Sanskrit, the plant figures prominently in the classical Ayurvedic canon such as Charaka Samhita, Sushruta Samhita and Ashtanga Hridayam, and is prescribed for complaints from oedema to calculi formation, skin disorders and retained placenta[2]. Its overlapping therapeutic role: under its Unani nomenclature Chirchira and multiple Siddha utilizations. In West and East Africa, where it flourishes as a common weed, practitioners from entirely different cultural traditions harness its roots, leaves and seeds to treat wounds, dental health, dysentery and hypertension; they also use it as an antidote to venomous bites [3]. The extent of this ethnopharmacological consensus is in itself a robust cue of true bioactivity.

A. aspera is an erect annual or short-lived perennial herb or undershrub reaching 0.3–1.5 m in height botanically. Its characteristic morphological attributes include opposite ovate to broadly elliptic leaves (3–12 cm long), terminal cylindrical spikes of small greenish white flowers, and prostatating reflexed fruiting bracts which derive both its common English name — prickly chaff flower — and its formidable dispersal potential. It occupies disturbed habitats with promiscuous ease: roadsides, field margins, waste ground throughout the pantropics and warm-temperate zones[4]. Commonly brushed aside as an agricultural pest, its healing properties far outweigh its negativity.

The systematic chemical examination of A. aspera, which started in earnest only in the 1950s, has uncovered a remarkable diversity in its phytochemical landscape[5]. Pharmacological profiles associated with these compound classes at the preclinical level so far have been anti-inflammatory, antioxidant, antidiabetic, antimicrobial, hepatoprotective, diuretic and cardioprotective/immunomodulatory. The biosynthetic programming of secondary metabolism varies enormously among organs: roots house defence compounds and structural building-blocks appropriate for the edaphic habitat; leaves store photoprotective flavonoids and photosynthetically-generated terpenoids; seeds generate storage lipids, nitrogen-rich toxic alkaloids, and germination-promoting saponins; flowers deploy pigments and volatile attractions. Charting these distributions is a prerequisite for making rational choices of plant material to develop herbal products, designing targeted extraction protocols, and determining which organs should take priority during bioactivity-guided fractionation[6]. It follows that the current study was planned to carry out a comprehensive, internally consistent, multi-organ phytochemical study on aerial parts of A. aspera using a freshly standardized multi-way solvent sequential extraction protocol, complete qualitative and quantitative phytochemical screening coupled with silica gel column chromatographic isolation and detailed spectroscopic characterization of major isolated compounds.

Materials and Methods:

Plant Collection, Authentication and Voucher Deposition: Fresh, morphologically intact specimens of A. aspera in the peak flowering and early fruiting stage were collected from five different collection sites within a radius range of 30 km from Moradabad. Specimen collection was scheduled for the post-monsoon phase (October–November) because published results show that this species has maximal secondary metabolite concentrations in its reproductive stage [7]. All five organs (i.e., roots, stems, leaves, flower including mature inflorescence bracts and mature seeds) were manually removed with sterile stainless-steel tools immediately after collection to avoid chemical cross-contamination among parts.

Preparation and Pre-treatment of Vegetal Material: Each organ was sequentially cleaned in three changes of tap water and then rinsed twice in deionized water to remove adhering soil, surface

microorganisms, and exogenous chemical residues. the material were spread in a single layer on clean muslin cloth and it was shade-dried at room temperature (25–32°C) under sufficient ventilating condition until the constant mass was reached over 14–21 days of period, which have been confirmed by weighing the sample after every 48 hours. This mild drying pathway was purposely selected in lieu of oven drying (degrading thermolabile flavonoids and phenolics) and lyophilization (reserved for specific volatile analysis). The dried material was ground to a fine powder using mechanical grinding. The powders were kept in amber-glass airtight containers at room temperature (20–25°C) and shielded from light until the extraction process.[8-13]

Sequential Polarity-Gradient Extraction: Sequential solvent extraction relies on the principle that solvents with different polarities selectively solubilize phytoconstituents with similar polarity, resulting in preliminary fractionation of the chemical mixture. Aliquots (100.0±0.1 g) of each powder were accurately weighed and macerated in increasing order of solvent polarity sequentially using the following five different solvents: petroleum ether (60–80 °C fraction), chloroform, ethyl acetate, methanol (95%), and distilled water. The maceration process was performed in three units of 24 hours, under room temperature and afterwards the slurry was vacuum filtered. We only transferred the residue from a cycle to the next solvent once we had filtered the previous extract and spread its marc — air-drying it for 2 h — so as to reduce carry-over of a previous solvent. A yield was given as % w/w dry plant material.

To facilitate direct comparison of the extraction efficiencies, leaf powder (the richest source of polar metabolites) was subjected to parallel extraction by Soxhlet extraction (6 h per solvent), UAE (50% Ethanol, 40 kHz, 50°C for 45 min) and MAE (70% Methanol:400 W:100°C for 12 min). Yield data were analyzed by one-way ANOVA followed by Tukey post-hoc comparison (p 10 min indicated as strong positive)

Phytochemical Screening: Phytochemical profiling was conducted using all extracts with Liebermann-Burchard reaction; terpenoids and steroids Salkowski & Liebermann-Burchard tests (with differentiation of triterpene (red) from steroid (green/blue) coloration); phenolic compounds ferric chloride, cardiac glycosides Keller-Killiani deoxy sugars for the test performance; coumarin Emerson's test, anthraquinones Borntrager's reaction; reducing sugars Fehling's then Benedicts. Results were recorded as strong positive (+++), moderate positive (++), weak positive (+), trace (±) or negative (–).[14].

Thin-Layer Chromatography Fingerprinting: TLC using silica gel 60 F254 pre-coated aluminium plates (Merck KGaA, Germany) was used for fingerprinting and to elute fractions from column chromatography according to elution profile. There were four solvent systems that were utilized: (i) EtOAc:MeOH:H2O (100:16.5:13.5 v/v), to separate flavonoids and phenolics; (ii) EtOAc:HCOOH:HAc:H2O (100:11:11:26 v/v), for hydroxycinnamic acid derivatives; (iii) CHCl3:MeOH:NH3 (85:14:1, v/v), for alkaloids; and 4) petroleum ether :EtOAc(7 :3,v/v), for terpenoids and phytosterols. Plates, prepared using twin-trough chambers pre-saturated with mobile phase vapour for 20 m, were air-dried (visualized at 254 nm (dark quenching) and 366 nm (fluorescence)) and sequentially sprayed by Natural Products/PEG reagent (flavonoids), Dragendorff's reagent (alkaloids), vanillin-sulfuric acid (terpenoids/steroids), and anisaldehyde-sulfuric acid respectively ca. the same day following development. Provisional identification was made by co-chromatography with authentic standards. Rf values were determined from at least three independent experiments and expressed as mean ± 0.02 [15].

Column Chromatographic Isolation: Preparative column chromatography (CC) was the main technique used for separating large amounts of major phytoconstituents. For all normal-phase separations, silica gel 60 (mesh 60–120, Merck) was used as stationary phase. For each of the plant parts, extracts were selected based on (i) having the most complex TLC fingerprint and/or (ii) highest content of target compound classes: roots- methanolic extract (alkaloids, ecdysteroids and saponins), leaves and

flowers - methanolic extract (flavonoids and phenolics), roots- petroleum ether extract (triterpenoids and sterols), seeds - methanolic extract (saponins and fatty acid derivatives). The homogenized protein sample was loaded by pre-adsorbing it onto silica gel (sample:silica 1:3 w/w) for equal band loading. Gradient elution was carried out over 12–18 h with binary and ternary solvent systems that progressed from 100% petroleum ether through incremental additions of ethyl acetate to 100% ethyl acetate, and then through ethyl acetate:methanol gradients to 100% methanol. Aliquots of 10–15 mL fractions were collected, monitored by TLC and subsequently pooled if similar profiles. Dried pooled fractions were further purified by repetitive recrystallization or flash chromatography until a purity of ≥95% was obtained as confirmed by HPLC peak area[16].

Spectroscopic Structure Elucidation: UV-Vis spectra (200–700 nm) were obtained in methanol or phosphate buffer as appropriate on a Shimadzu UV-1800 spectrophotometer with and without diagnostic shift reagents for analysis of flavonoid substitution patterns (NaOMe, AlCl3, NaOAc, and H3BO3). As a cross-check, FTIR spectra were obtained using attenuated total reflectance (ATR) mode with KBr pellets on a Bruker ALPHA spectrometer (400–4000 cm^{-1}). The 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 spectrometer in DMSO-d6 or CDCl3 using TMS as the internal reference. Where necessary, 2D experiments (COSY, HSQC, HMBC) were used for full proton and carbon assignment. HRMS data were obtained on a Waters SYNAPT G2-Si instrument with ESI ionization in positive and negative modes; molecular formulae were confirmed from accurate mass measurements (error < 2 ppm). Structures were confirmed by comparison with published NMR and MS data and, where available, with authenticated reference standards using TLC co-chromatography[17].

Result:

Table 1: Comparative extraction yields (% w/w dry plant material, mean ± SD, n = 3) obtained from various plant parts of *Achyranthes aspera Linn.* using different extraction methods and solvents.

Extraction Method / Solvent	Roots (%)	Stems (%)	Leaves (%)	Seeds (%)	Flowers (%)
Maceration / Methanol	17.1 ± 1.3	14.5 ± 1.4	22.2 ± 1.8	11.7 ± 1.2	21.1 ± 1.5
Soxhlet / Pet. Ether	6.2 ± 0.5	3.1 ± 0.4	5.2 ± 0.8	9.2 ± 0.8	5.6 ± 0.6
Soxhlet / Chloroform	7.6 ± 0.7	6.2 ± 0.6	6.3 ± 0.7	8.7 ± 1.2	5.6 ± 0.7
Soxhlet / Ethyl Acetate	09.4 ± 1.0	7.9 ± 1.1	11.7 ± 1.2	8.1 ± 0.7	12.3 ± 0.6
Soxhlet / Methanol	21.6 ± 1.6	15.2 ± 1.5	23.7 ± 1.7	15.1 ± 1.0	21.7 ± 1.8
UAE / Ethanol (50%)	21.3 ± 1.4	16.2 ± 1.6	27.1 ± 1.2	14.3 ± 1.1	22.3 ± 1.4
MAE / Methanol (70%)	23.2 ± 1.6	21.7 ± 1.5	30.1 ± 1.6	10.2 ± 1.6	27.1 ± 1.2
Aqueous / Hot infusion	12.4 ± 1.1	10.2 ± 1.0	14.7 ± 1.2	8.6 ± 0.7	13.7 ± 1.2

UAE = Ultrasonic-Assisted Extraction; MAE = Microwave-Assisted Extraction; values within a row followed by different superscript letters differ significantly ($p < 0.05$, Tukey HSD test).

Extraction Yield and Method Comparison: Extraction yields varied substantially across plant parts, solvents, and methods. Comprehensive yield data for all combinations investigated are presented in Table 1. Across all plant parts and conventional Soxhlet extraction, methanolic extracts consistently afforded the highest yields, confirming the predominance of medium-to-high-polarity secondary metabolites in all organs of A. aspera. Petroleum ether extracts were consistently lowest (6.2–9.2%),

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with seeds yielding the most non-polar material, reflecting their high lipid content. Aqueous hot infusion yielded 8.6–14.7%, with leaves and flowers returning the highest values owing to their high content of water-soluble polyphenols and oligosaccharides

Qualitative Phytochemical Screening: Qualitative screening of all extracts from five plant parts of A. aspera is summarized in Table 2. Alkaloids were detected in all plant parts using Mayer's, Dragendorff's, and Wagner's tests, with the most intense positive reactions (precipitate formation within 30 seconds) observed in chloroform and methanolic extracts of roots and seeds. The dual positivity across both non-polar and polar solvents is consistent with the presence of both lipophilic (achyranthine) and highly polar (betaine) alkaloid types. Flavonoids gave strong positive reactions in leaves and flowers, evidenced by intense magenta-crimson coloration in the Shinoda test and bright yellow-orange fluorescence at 366 nm following Natural Products/PEG spray on TLC plates, with these results strongest in ethyl acetate and methanolic fractions of these organs.

Saponins were identified by positive froth tests (foam persisting >15 min, indicating high saponin content) and Liebermann-Burchard reactions in root and seed extracts. The transient pinkish-red color with Liebermann-Burchard reagent indicated triterpenoid rather than steroidal saponins as the primary saponin type. Tannins were broadly distributed but most concentrated in stems and leaves (as expected from their role in deterring herbivory in photosynthetically active tissue), giving dark blue-black precipitates with ferric chloride. Terpenoids and steroids gave strongly positive Salkowski and Liebermann-Burchard reactions in petroleum ether extracts of all parts, with roots and seeds yielding the most intense colorations. Cardiac glycosides were detected by the Keller-Killiani test specifically in root and stem methanolic extracts, while phenolic compounds were universally present. Coumarins were detected only as trace signals in leaf and flower fractions, and anthraquinones were essentially absent.

Table 2: Qualitative phytochemical screening of extracts from various parts of *Achyranthes aspera Linn*. (+++) strongly positive; (++) moderately positive; (+) weakly positive; (±) trace; (–) absent.

Phytochemical Class	Roots	Stems	Leaves	Seeds	Flowers
Alkaloids	+++	++	++	+++	+
Flavonoids	+	++	+++	+	+++
Saponins	+++	++	+	+++	+
Tannins	+	++	+++	+	++
Terpenoids	+++	++	++	+++	+
Phenolic compounds	++	++	+++	+	+++
Steroids	+++	++	+	+++	+
Cardiac glycosides	++	+	+	-	-
Reducing sugars	+	+	++	+	++
Coumarins	+/-	-	+/-	-	+
Anthraquinones	-	-	+/-	-	-

Results represent consensus of Mayer's, Dragendorff's, and Wagner's tests (alkaloids); Shinoda test and alkaline reagent (flavonoids); froth test and Liebermann-Burchard (saponins); FeCl3 and

lead acetate (tannins); Salkowski and Liebermann-Burchard (terpenoids); Keller-Killiani test (cardiac glycosides).

TLC Fingerprinting and Rf Profiles: TLF generated reproducible, organ-specific chromatographic profiles that were standardization references and column chromatography guidance. The most complicated profiles, with the highest concentration of information were obtained from methanol extracts of leaves and flowers, yielding an average of 10–14 distinct bands per plate in the flavonoid/phenolics solvent system compared to only 5–8 bands in those prepared from root and seed extracts. Table 3 contains all Rf data for the identified compounds. NP/PEG spray was followed by the extract of leaves and flowers exhibiting intense bright-yellow fluorescent spots (EtOAc:MeOH:H2O, 100:16.5:13.5) under UV265 nm which were identified as rutin and quercetin respectively through co-chromatography with respective authentic reference standards. The root extracts developed prominent bright orange precipitate spots (CHCl3:MeOH:NH3, 85:14:1) in the alkaloid system with Dragendorff's reagent, corresponding to betaine and achyranthine respectively. This fingerprinting provides a reliable set of quality-control reference data for standardization of A. aspera-derived herbal preparations.

Table 3: TLC fingerprint data for major identified compounds from *Achyranthes aspera Linn*. extracts. Rf values represent means from three independent TLC runs (±0.02). NP/PEG = Natural Products/Polyethylene glycol spray reagent.

Compound	Chemical Class	Mobile Phase System	Rf Value	UV (366 nm)	Spray Reagent Color
Rutin	Flavonoid	EtOAc:MeOH:H_2O (100:16.5:13.5)	0.33	Yellow-green	NP/PEG: Bright yellow
Quercetin	Flavonoid	EtOAc:MeOH:H_2O (100:16.5:13.5)	0.45	Yellow-orange	NP/PEG: Orange
Chlorogenic acid	Phenolic acid	EtOAc:HCOOH:HAc:H_2O (100:11:11:26)	0.49	Blue-white	FeCl3: Dark green
Caffeic acid	Phenolic acid	EtOAc:HCOOH:HAc:H_2O (100:11:11:26)	0.42	Blue	FeCl3: Dark blue
Betaine	Alkaloid	$CHCl_3$:MeOH:NH_3 (85:14:1)	0.29	Dark quench	Dragendorff: Orange
Achyranthine	Alkaloid	$CHCl_3$:MeOH:NH_3 (85:14:1)	0.63	Dark quench	Dragendorff: Orange-red
Ecdysterone	Ecdysteroid	$CHCl_3$:MeOH (9:1)	0.74	Blue-purple	Vanillin-H2SO4: Violet
Oleanolic acid	Triterpenoid	Pet. ether:EtOAc (7:3)	0.69	Quench	Anisaldehyde: Pink
Beta-sitosterol	Phytosterol	Pet. ether:EtOAc (7:3)	0.75	Quench	Vanillin-H2SO4: Blue

Isolation and Spectroscopical Characterization of Main Compounds: Column chromatographic separation of methanolic and petroleum ether extracts of all five plant parts of A. aspera resulted in fourteen characterized compounds. The table lists their identities, sources plant parts, isolation techniques,

molecular weights, yields and main biological functions (Table 4). Summary of selected spectroscopic data for relevant isolates are shown below.

Table 4: Bioactive compounds isolated from various parts of *Achyranthes aspera Linn*. by column chromatography, with spectroscopic identity confirmation and reported biological activities. All yields expressed per 100 g dry plant material. Purity ≥ 95% by HPLC peak area.

Compound	Chemical Class	Plant Part	MW (Da)	Isolation Method	Key Activity	Yield (mg/100g)
Betaine	Alkaloid	Roots, Seeds	117	Ion-exchange CC	Hepatoprotective, Osmoprotectant	98
Achyranthine	Alkaloid	Roots	198	Silica CC/HPLC	Antihypertensive, Analgesic	110
Ecdysterone	Ecdysteroid	Roots, Leaves	480	Prep-HPLC	Anabolic, Anti-inflammatory	36
Ecdysone	Ecdysteroid	Roots	464	Prep-HPLC	Immunomodulatory	8
Oleanolic acid	Triterpenoid	All parts	456	Silica CC	Anti-inflammatory, Hepatoprotect.	23
Rutin	Flavonoid	Leaves, Flowers	610	HPTLC/Prep-HPLC	Cardioprotective, Antioxidant	37
Quercetin	Flavonoid	Leaves	302	Silica CC	Anti-inflammatory, Antidiabetic	16
Caffeic acid	Phenolic acid	Leaves, Flowers	180	RP-HPLC	Antioxidant, Antimicrobial	8
Chlorogenic acid	Phenolic acid	Leaves	354	RP-HPLC	Hypoglycemic, Antioxidant	6
Beta-sitosterol	Phytosterol	Seeds, Roots	414	Silica CC	Anticancer, Hypolipidemic	15
Lupeol	Pentacyclic terp.	Roots, Stems	426	Silica CC	Anti-arthritic, Antiprotozoal	12

Discussion The combined multitechnic, multi-organ study presented here represents the most exhaustive comparative phytochemical profiling of Achyranthes aspera to date. By treats five different plant parts of a single species under identical extraction protocols, analytical methods and statistical treatments simultaneously, we produce an internally consistent chemical dataset that overcomes the major shortcomings of previous single-part or -class studies. The following discussion tries to consolidate the overall findings and their consequences to traditional medicine, drug discovery, standardization science and future research steps.

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