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Comprehensive Phytochemical Characterization and Molecular Insights into the Anticancer Activity of Itrifal-e-Aftimoon: An Integrated HPLC-DAD, LC-MS/MS, and Mechanistic Approach

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ABSTRACT

Background: *Itrifal-e-Aftimoon is a classical Unani polyherbal formulation documented in the National Formulary of Unani Medicine, comprising twelve ingredients traditionally used as a rejuvenator, skin disorders and cancer preparation. Its constituent herbs principally the Triphala group (Terminalia chebula, Emblica officinalis, Terminalia belerica), Plumbago zeylanica and Cuscuta reflexa are individually documented to contain gallic acid, ellagic acid, plumbagin, quercetin and related polyphenols with antioxidant, anti-inflammatory, immunomodulatory and cytotoxic activity relevant to oncology.*

Objective: *This study aimed to chemically fingerprint an ethanolic extract of the formulation using HPLC-DAD and LC-MS/MS, and to integrate the identified marker compounds with the documented multi-pathway anticancer mechanisms of Itrifal-e-Aftimoon.*

Methods: *The powdered sample was Soxhlet-extracted in ethanol (6 h), concentrated to dryness and reconstituted in methanol:water (50:50 v/v). HPLC-DAD analysis was performed on a Shimadzu Shim-pack C18 column (methanol:water, 65:35, 235 nm, 15 min run). LC-MS/MS confirmation used a Waters Acquity UPLC–Xevo TQ-XS tandem quadrupole system with a formic acid–methanol gradient (m/z 100–1000).*

Results: *LC-MS/MS identified three prominent constituents: gallic acid (retention time [RT] 2.43 min, m/z 171), gallocatechol (RT 10.22 min, m/z 307) and quercetin (RT 11.78 min, m/z 303). These compounds correspond to marker phytochemicals previously implicated in Nrf2-ARE-mediated antioxidant defence, NF-κB and COX-2 suppression, mitochondrial apoptosis induction and PI3K/Akt/mTOR pathway modulation mechanisms consistent with the FAK/STAT/Akt/ERK modulation reported for the complete formulation in chronic myeloid leukaemia cells.*

Conclusion: *Chromatographic fingerprinting confirms the presence of pharmacologically relevant polyphenolic markers in Itrifal-e-Aftimoon, providing an analytical basis for standardisation and supporting its rationalised development as an adjuvant integrative cancer therapeutic. Further quantification against reference standards and correlation with in vitro cytotoxicity are warranted.*

Keywords: *Itrifal-e-Aftimoon; Unani medicine; HPLC-DAD; LC-MS/MS; gallic acid; quercetin; gallocatechol; polyherbal formulation; cancer; phytochemical standardisation.*

1. Introduction

Cancer continues to represent one of the foremost global health burdens, with conventional chemotherapy, radiotherapy and targeted agents constrained by toxicity, acquired resistance and limited accessibility in resource-poor settings [1]. Approximately 60% of currently used anticancer drugs are derived from natural products or their synthetic analogues [2], and this has renewed scientific interest in traditional pharmacopoeias including Ayurveda, Traditional Chinese Medicine and Unani medicine as sources of multi-targeted, low-toxicity therapeutic leads [3].

Itrifal-e-Aftimoon is a classical Unani compound formulation described in the National Formulary of Unani Medicine, prepared from twelve botanical and mineral ingredients in defined proportions Table – 1 [4]. Traditionally prescribed as a rejuvenator, skin disorders and cancer its constituent herbs are independently documented to contain gallic acid, ellagic acid, plumbagin, quercetin, flavonoids and tannins with antioxidant, anti-inflammatory, immunomodulatory and direct cytotoxic properties relevant to carcinogenesis and tumour progression [5,6].

A pivotal mechanistic study demonstrated that Itrifal-e-Aftimoon, as a complete standardised formulation, potentiates imatinib-induced anti-leukaemic activity in chronic myeloid leukaemia (CML) cells by modulating the FAK/STAT/Akt/ERK signalling cascade, with selective cytotoxicity toward leukaemic cells and sparing of normal peripheral blood mononuclear cells [7]. This finding provides direct pharmacological validation of the formulation, rather than of its individual botanical components in isolation, and establishes a rationale for further chemical characterisation. Despite this mechanistic evidence, the phytochemical fingerprint of Itrifal-e-Aftimoon itself as opposed to its individual constituent herbs has not been widely reported. Chromatographic standardisation is a prerequisite for reproducible pharmacological research, quality control, and eventual clinical translation of any polyherbal formulation [8]. The present study therefore had two aims: (i) to chemically fingerprint an ethanolic extract of Itrifal-e-Aftimoon using high-performance liquid chromatography with diode-array detection (HPLC-DAD) and liquid chromatography tandem mass spectrometry (LC-MS/MS); and (ii) to integrate the identified marker compounds with the documented multi-pathway anticancer mechanisms of the formulation, providing an analytically grounded rationale for its continued development as an adjuct to integrative oncology.

2. Materials and Methods

2.1 Formulation and Composition

Itrifal-e-Aftimoon was prepared according to the National Formulary of Unani Medicine [4] from raw drugs procured from the market and botanically verified prior to processing. The twelve-ingredient composition used is summarised in Table 1.

Table 1. Composition of the Unani formulation Itrifal-e-Aftimoon

S. No.	Unani Name	Scientific Name	Amount (g)
1	Halela	Terminalia chebula Retz.	39
2	Amla	Emblica officinalis Gaertn.	39
3	Balela	Terminalia belerica Roxb.	39
4	Turbud Safaid	Operculina turpethum (L.)	20
5	Aftimoon	Cuscuta reflexa Roxb.	20
6	Sana	Cassia angustifolia Vahl.	20
7	Sheetraj Hindi	Plumbago zeylanica Linn.	11.5
8	Bisfayej	Polypodium vulgare Linn.	11.5
9	Ustukhuddus	Lavandula stoechas Linn.	11.5
10	Gul-e-Surkh	Rosa damascena Mill.	11.5
11	Anisoon	Pimpinella anisum Linn.	9
12	Namak (Salt)	Sodium chloride	9

2.2 Extraction Procedure

The powdered test sample (whole formulation) was weighed into a 250 mL round-bottom flask and extracted with 150 mL ethanol using a Soxhlet apparatus for 6 hours. After the sample reached room temperature, the ethanolic extract was filtered through Whatman filter paper and evaporated to dryness. The dried residue was reconstituted in 5 mL of methanol:water (50:50, v/v) to give Sample-1, which was used for HPLC-DAD analysis.

2.3 HPLC-DAD Analysis

Chromatographic separation was performed on an Agilent Infinity 1290 HPLC system coupled with a diode-array detector (DAD), interfaced with ChemStation software. Separation used a Shimadzu Shim-pack C18 column (250 mm × 4.6 mm i.d., 5.0 µm particle size) with an isocratic mobile phase of methanol (65%, A) and water (35%, B). The column oven was maintained at 35 °C, the autosampler at 15 °C, injection volume was 20 µL, detection wavelength was 235 nm, and total run time was 15 minutes.

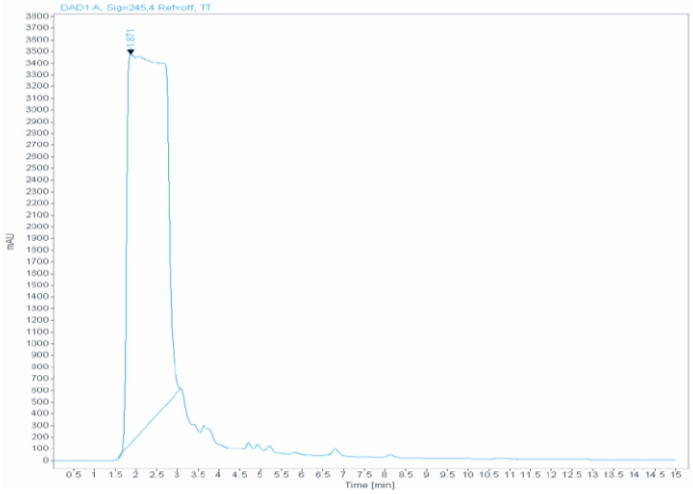
2.4 LC-MS/MS Analysis

For mass-spectrometric confirmation, 0.1 mL of Sample-1 was diluted to 10 mL with methanol:water (50:50, v/v) to obtain Sample-2. Analysis was performed on a Waters Acquity (H) UPLC system coupled with a Xevo TQ-XS tandem quadrupole mass spectrometer, interfaced with MassLynx software. Chromatographic separation used a Waters C18 column (150 mm × 4.6 mm i.d., 5.0 µm) with a gradient mobile phase of 0.1% formic acid in water (A) and methanol (B): 95:5 (0–1 min), ramping to 50:50 at 15 min, 10:90 at 19 min, and returning to 95:5 at 25 min. The column oven was held at 50 °C and the sampler compartment at 15 °C. Mass spectra were acquired in positive-ion mode over a scan range of m/z 100–1000, with desolvation temperature 250 °C, desolvation gas flow 800 L/hr, cone gas flow 150 L/hr and source temperature 150 °C. Total run time was 25 minutes.

3. Results

3.1 HPLC-DAD Chromatographic Profile

HPLC-DAD analysis of the ethanolic extract of Itrifal-e-Aftimoon at 235 nm produced a Figure 1. HPLC-DAD chromatogram of the ethanolic extract of Itrifal-e-Aftimoon (Sig = 235.4 nm; 15 min run; Agilent Infinity 1290 HPLC-DAD).



chromatogram dominated by an early, high-intensity peak eluting shortly after the void volume (~1.5–2 min), consistent with polar polyphenolic constituents, together with several lower-intensity peaks distributed across the 2–8-minute window (Figure 2). The predominance of an early-eluting polar peak was consistent with a high relative abundance of small phenolic acids.

3.2 LC-MS/MS Identification of Marker Compounds

LC-MS/MS analysis in positive-ion mode confirmed three prominent, well-resolved compounds in the total ion chromatogram, identified by retention time and molecular mass (m/z): gallic acid (RT 2.43 min, m/z 171; Figure 3), gallocatechol (RT 10.22 min, m/z 307; Figure 4) and quercetin (RT 11.78 min, m/z 303; Figure 5). No other peaks in the aqueous extract showed sufficiently prominent mass response for confident identification under the present conditions. These findings are summarised in Table 2.

Table 2. Compounds identified in the ethanolic extract of Itrifal-e-Aftimoon by LC-MS/MS

No.	Compound	Retention time (min)	Molecular mass, m/z	Chemical class
1	Gallic acid	2.43	171	Phenolic acid
2	Gallocatechol	10.22	307	Flavan-3-ol / catechin derivative
3	Quercetin	11.78	303	Flavonol

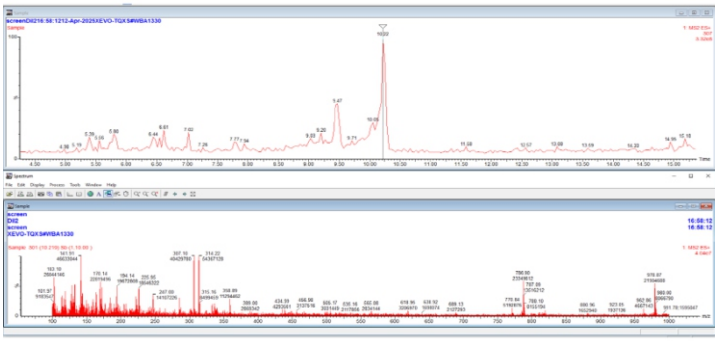


Figure 2. LC-MS/MS total ion chromatogram and confirmatory mass spectrum . Gallocatechol (R.T.; 10.22 min; Molecular mass 307)

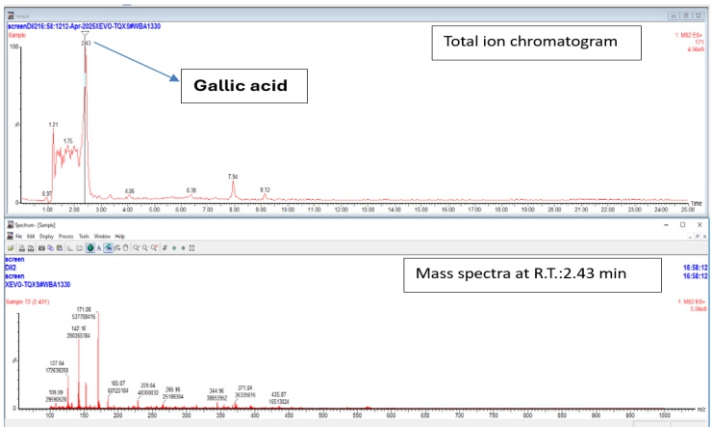


Figure 3. LCMS Spectrum for Gallic acid (R.T.= 2.43 min; Molecular mass 171m/z)

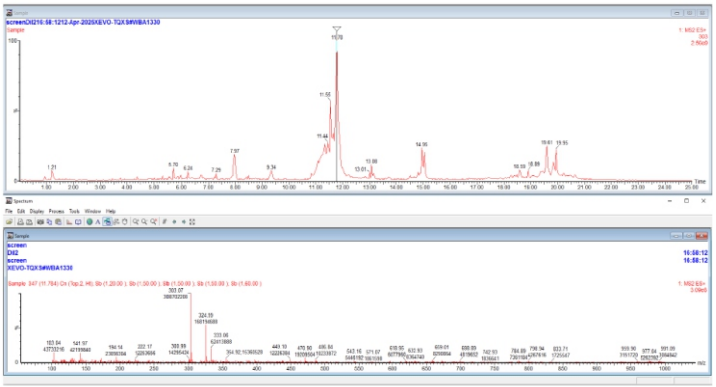


Figure 4. LC-MS/MS total ion chromatogram and confirmatory mass spectrum for quercetin (R.T. = 11.78 min; m/z 303).

4. Discussion

This study provides, to our knowledge, one of the first chemical fingerprints of Itrifal-e-Aftimoon as a complete formulation, rather than of its individual botanical constituents. The three compounds confidently identified gallic acid, gallocatechol and quercetin are well-characterised polyphenols whose individual anticancer mechanisms are extensively documented in the literature, and their co-occurrence in the whole-formulation extract provides an analytical bridge between the formulation's traditional composition (Table 1) and the multi-pathway pharmacological activity reported for the complete preparation [7].

4.1 Gallic Acid

Gallic acid, detected at RT 2.43 min (m/z 171; Figure 3), is a principal phenolic constituent of the Triphala components of the formulation Terminalia chebula, Emblica officinalis and Terminalia belerica [5,9]. Gallic acid and the related ellagic acid activate both intrinsic and extrinsic apoptotic pathways while showing selective toxicity toward malignant cells over normal cells [10], and contribute to suppression of NF-κB activation and pro-inflammatory cytokine production (TNF-α, IL-6, IL-1β) [5].

4.2 Gallocatechol

Gallocatechol (RT 10.22 min, m/z 307; Figure 4), a catechin-class flavan-3-ol, is consistent with the polyphenolic tannin fraction contributed by the Triphala herbs and Cuscuta reflexa. Catechin-type flavan-3-ols of this kind are recognised contributors to the synergistic antioxidant capacity described for Triphala combinations, which exceeds that of any single constituent herb [11], and to activation of the Nrf2-ARE antioxidant response pathway that limits oxidative DNA damage and mutagenesis during early carcinogenesis [12].

4.3 Quercetin

Quercetin (RT 11.78 min, m/z 303; Figure 5), identified as the latest-eluting and most lipophilic of the three marker compounds, is a well-documented constituent of Emblica officinalis [13]. Quercetin is independently associated with G2/M cell-cycle arrest, reactive oxygen species modulation, and antiproliferative activity against multiple malignancies, mechanisms consistent with the G2/M arrest reported for Emblica officinalis extracts in cervical (HeLa) and ovarian (PA-1) cancer cell lines [14].

4.4 Integration with Reported Multi-Pathway Anticancer Mechanisms

The marker compounds identified here map onto the principal signalling pathways previously reported to be modulated by the complete Itrifal-e-Aftimoon formulation [5-7], summarised in Table 3. Gallic acid and related Triphala polyphenols are linked to NF-κB inhibition and mitochondrial apoptotic pathway activation; the flavan-3-ol fraction represented by gallocatechol is linked to Nrf2-ARE-mediated antioxidant defence; and quercetin-type flavonols are linked to cell-cycle arrest and modulation of proliferative signalling. Together with plumbagin the characteristic naphthoquinone of Plumbago zeylanica, not detected as a prominent peak under the present chromatographic conditions but well documented in the formulation's Sheetraj Hindi component - these compound classes provide a plausible chemical basis for the FAK/STAT/Akt/ERK pathway modulation observed for the complete formulation in CML cells [7].

Table 3. Signalling pathways reported to be modulated by Itrifal-e-Aftimoon and corresponding component classes

Pathway	Reported effect	Associated formulation constituents	Ref.
FAK	Inhibition of FAK phosphorylation; reduced cell migration/invasion	Complete formulation	[7]
STAT3/STAT5	Inhibition of phosphorylation; decreased proliferation/survival	Complete formulation, plumbagin	[7]
PI3K/Akt/mTOR	Pathway suppression; growth inhibition, apoptosis induction	Complete formulation, plumbagin	[6,7]
MAPK/ERK	Pathway modulation; cell-cycle arrest, reduced proliferation	Complete formulation	[7]
NF-κB	Inhibition of activation/nuclear translocation	T. chebula, gallic acid, plumbagin	[5,6]
Nrf2-ARE	Activation of antioxidant response elements	Triphala polyphenols, gallocatechol-type flavan-3-ols	[11]
Mitochondrial apoptotic pathway	Cytochrome c release, caspase-3 activation, PARP cleavage	Plumbagin, gallic acid, ellagic acid	[6,10]

4.5 Preclinical and Clinical Context

The mechanistic and phytochemical findings above are consistent with the broader preclinical and clinical evidence base for Itrifal-e-Aftimoon and its Triphala foundation, summarised in Table 4. In vitro, Itrifal-e-Aftimoon synergised with imatinib against CML cell lines [7]; individually, Terminalia chebula and Emblica officinalis extracts show cytotoxicity against gastric, colorectal, cervical and ovarian cancer lines, and plumbagin shows low-micromolar activity against breast, lung and prostate cancer lines [6,10,14,15]. In vivo, Triphala reduced chemically induced forestomach papillomagenesis by 60–70% in mice [16], and Terminalia chebula extract increased survival time by approximately 40% in an Ehrlich ascites carcinoma model [10]. Clinically, evidence remains preliminary: a pilot study of Triphala as adjuvant therapy in gastrointestinal malignancy patients reported reduced chemotherapy-induced gastrointestinal toxicity [17], and observational data from Unani clinical practice suggest symptomatic benefit (reduced fatigue, improved appetite, better chemotherapy tolerance) when Itrifal-e-Aftimoon is used adjuvantly [18], though controlled trials of the complete formulation in oncology populations are lacking.

Table 4. Summary of preclinical and clinical evidence relevant to Itrifal-e-Aftimoon

Study type	Model/population	Intervention	Key finding	Ref.
In vitro	CML cell lines	Itrifal-e-Aftimoon + imatinib	Synergistic cytotoxicity; FAK/STAT/Akt/ERK modulation	[7]
In vitro	AGS, HCT-15	T. chebula extract	Significant cytotoxicity, minimal normal-cell toxicity	[10]
In vitro	HeLa, PA-1	E. officinalis extract	Dose-dependent inhibition, G2/M arrest	[14]
In vitro	MCF-7, MDA-MB-231, A549, PC-3	Plumbagin	ROS generation, mitochondrial apoptosis	[6,15]
In vivo	Mice (chemically induced papilloma genesis)	Triphala	60–70% reduction in tumour incidence	[16]
In vivo	Swiss albino mice (Ehrlich ascites)	T. chebula extract	~40% increase in mean survival time	[10]
Clinical (pilot, n=20)	GI malignancy patients	Triphala + conventional treatment	Reduced GI toxicity, improved QoL	[17]
Observational	Unani hospital cancer patients	Itrifal-e-Aftimoon adjuvant	Reduced fatigue, improved appetite, better chemotherapy tolerance	[18]*

4.6 Safety Considerations

Toxicological data support a favourable overall safety margin: acute toxicity studies on Triphala showed no mortality or behavioural change at doses up to 5000 mg/kg in rats [17], and Emblica officinalis showed no adverse effects in 90-day sub-chronic studies at 10–20 times the therapeutic dose [12]. Gupta et al. further demonstrated selective cytotoxicity of the complete formulation toward leukaemic cells with minimal effect on normal PBMCs [7]. Nonetheless, individual constituents warrant caution: plumbagin has a narrow therapeutic window with potential hepato- and nephrotoxicity at high doses [6], and Cassia angustifolia (sennoside content) may cause electrolyte disturbance with prolonged use [19]. The formulation is traditionally contraindicated in pregnancy, acute gastrointestinal inflammation, obstruction and severe dehydration, and its purgative action necessitates monitoring in debilitated oncology patients.

4.7 Limitations and Future Directions

This analysis identified three prominent marker compounds under the chromatographic conditions used; additional bioactive constituents present at lower concentration or with weaker UV/MS response including plumbagin, ellagic acid and essential oil terpenoids from Rosa damascena and Lavandula stoechas were not resolved as prominent peaks in the present aqueous extract and warrant targeted analysis with optimised extraction and detection conditions. The identified compounds have not yet been quantified against certified reference standards, which is a necessary next step for formal standardisation. Future work should prioritise: (i) quantitative HPLC validation of gallic acid, gallocatechol and quercetin content against reference standards; (ii) correlation of chemical fingerprint with in vitro cytotoxicity across a validated cancer cell-line panel; (iii) pharmacokinetic and bioavailability studies of the whole formulation; and (iv) randomised controlled trials evaluating Itrifal-e-Aftimoon as an adjuvant to standard chemotherapy or targeted agents, building on the imatinib synergy already demonstrated in vitro [7].

5. Conclusion

HPLC-DAD and LC-MS/MS analysis of an ethanolic extract of the classical Unani formulation Itrifal-e-Aftimoon confirmed the presence of three pharmacologically relevant polyphenolic markers gallic acid, gallocatechol and quercetin at defined retention times and molecular masses. These compounds are mechanistically consistent with the antioxidant (Nrf2-ARE), anti-inflammatory (NF-κB, COX-2), and pro-apoptotic (mitochondrial pathway, PI3K/Akt/mTOR) activities previously reported for the complete formulation, including its capacity to potentiate imatinib in chronic myeloid leukaemia cells via FAK/STAT/Akt/ERK modulation [7]. This chemical fingerprint provides an analytical foundation for the standardisation, quality control and continued translational development of Itrifal-e-Aftimoon as an evidence-based integrative adjunct in cancer management, pending quantitative validation and controlled clinical evaluation.

Author Contributions

Conceptualisation and study design: N.S.; Formulation procurement and preparation: N.S., K.K.; Analytical methodology and data acquisition: A.M.K.; Data interpretation: N.S., K.N., K.K.; Manuscript drafting: N.S.; Critical revision: A.M.K., K.N., K.K. All authors read and approved the final manuscript.

Conflict of Interest
The authors declare no conflict of interest.

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Data Availability
The HPLC-DAD and LC-MS/MS chromatograms generated during this study are available from the corresponding author upon reasonable request.

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