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# HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION AND QUANTITATION OF CONVENTIONAL UNANI FORMULATIONS

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**Abstract***:* For the simultaneous quantitative determination of nine bioactive compounds, including four phenolic (gallic acid, ellagic acid, chebulinic acid, and tannic acid), two flavanoids (rutin and quercetin), two anthraquinones (sennoside A and B), and one oxygenated hydrocarbon (vitamin C), a new High Performance Liquid Chromatography-Photodiode Array Detector (HPLC-PDA) method has been Gradient elution was used to separate the samples on a C18 LiChrospher 100 column (5 mm, 250 3 4.6 mm), and the results were recorded at 254 nm. The findings showed that the suggested method is reliable, precise, cost-effective, and appropriate for quality control of conventional polyherbal Unani formulations incorporating complex chemicals with various structures, such as Itrifals.

**Keywords:** Unani Medicine, Conventional Drugs, Polyherbal Formulation, HPLC–PDA, Quality control of Herbal Drugs.

**Introduction***:* Since the beginning of time, people have used herbal medicines to cure and prevent a variety of illnesses. The danger associated with herbal remedies is generally thought to be relatively low, but reports of serious reactions highlight the necessity for the creation of efficient marker systems for the separation and identification of the various components. Herbal medication standardization, stability, and quality control are possible but challenging to implement. The goal of the current work is to create a multi-component HPLC assay method that will aid in the quality control of herbal formulations.

The therapeutic effectiveness of conventional Unani multi-herbal formulations is thought to be an integrative outcome of numerous bioactive components. The several ingredients in the herbs and formulations may function "synergistically" to produce the best medicinal results. For the quality control of these kinds of formulations, simultaneous determination of the biologically active ingredients is therefore required.

According to the Unani system, Halela (*Terminalia chebula*), Balela (*Ter-minalia belerica*), and Amla are the three different types of fruits that make up Itrifal or Trifaloon (Emblica officinalis). Itrifals have been shown to have anti-oxidant properties, and are frequently used as a general body tonic since they are believed to be efficient at removing waste products from the body. The base formulation "Itrifal" may also contain other substances, such as *Itrifal-e-Aftimoon* (IA) and *Itrifal-e-Badiyan* (IB), which were chosen for the current investigation. The IA contained twelve crude drugs, including *T. chebula* (15.2% w/w), *T. belerica* (15.2% w/w), *E. officinalis* (15.2% w/w), Operculinaturpenthum (15.2% w/w), *Cuscutaepi-thymum* (7.4% w/w), *Cassia angustifolia* (4.5% w/w), *Plumbago zeylanica* (4.5% Both formulations share five ingredients: *T. chebula*, *T. belerica, E. officinalis, R. damascene, and P. amygdalus*; however, all Itrifals should primarily comprise *T. chebula, T. belerica, and E. officinalis*. Following the steps outlined in NFUM, two batches (Batch I and Batch II) for IA and IB were created in the lab.

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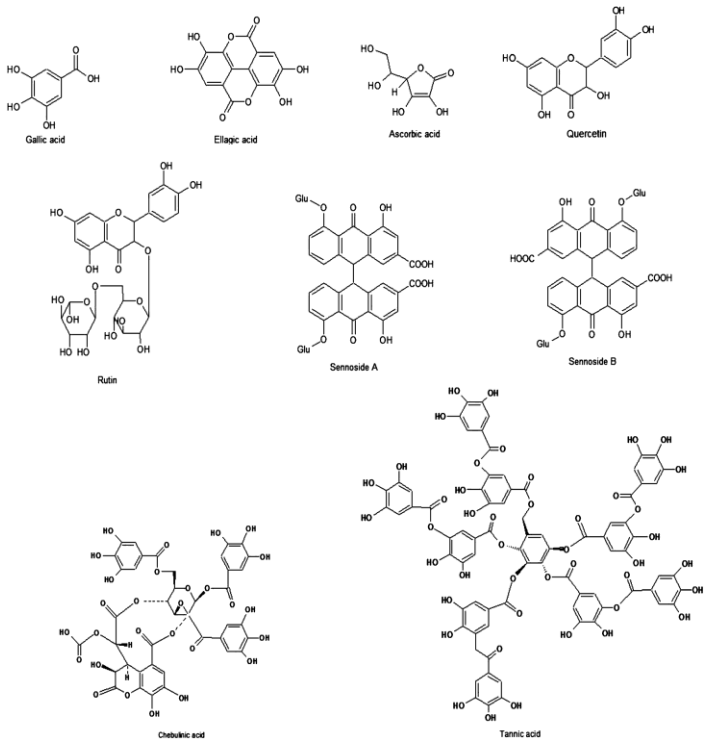
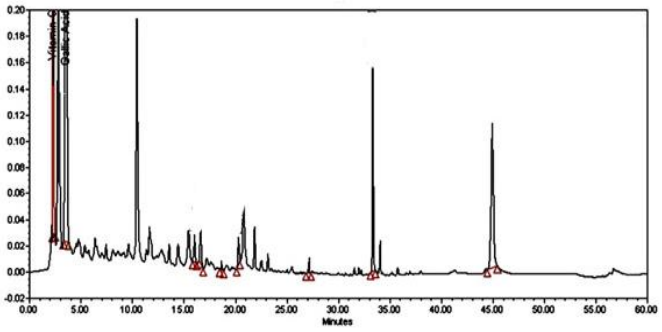
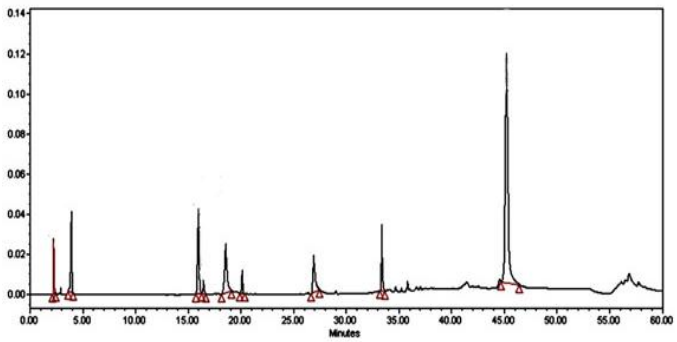



**Fig.1 Chemical structure of all bioactive compounds selected for analysis.**



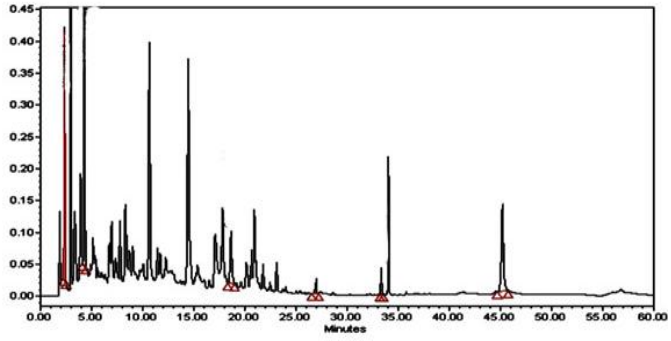
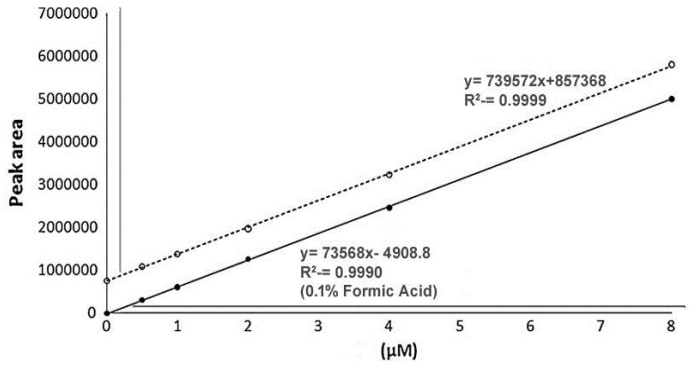




**Fig.2 Sample Itrifal-e-Aftimoon HPLC-PDA chromatogram at 254 nm showing chemicals eluting (a) the standard and (b) the sample (c) Sample from Itrifal-e-Badiyan**

500 g of sugar and 300 mL of water were combined to create concentrated sugar syrup, which was then boiled for 15 minutes while being constantly stirred. The sugar syrup was continuously stirred while the medications were sieved and ground up one at a time.

The primary anti-oxidants in these Itrifal formulations are free phenolic acids including ascorbic acid, gallic acid, ellagic acid, chebulinic acid, and tannic acid as well as free flavonoids like rutin and quercetin. Sennoside A and B, anthraquinone glycosides with a significant presence in IA, were chosen as a specific identifier for *C. angustifolia*.

A review of the literature reveals that, as of yet, no methodologies for the quantitative examination of several chemicals in Itrifal formulations have been published. The majority of earlier research used HPTLC, HPLC-CAD, HPLC-DAD, and HPLC-DAD-ESIMS to analyse the phenolic and flavonoid components in various herbal samples.



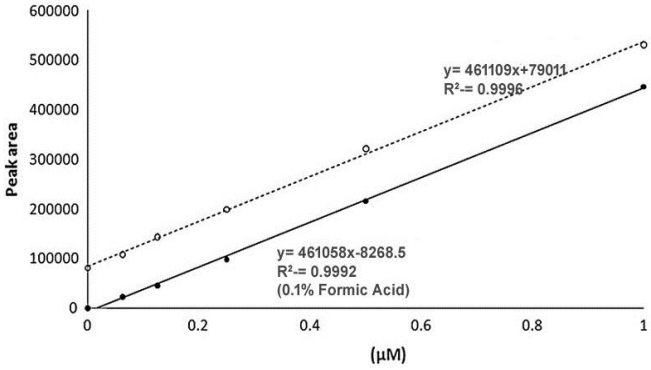



**Fig 3. Peak area and sample concentration have linear correlations with one another.**

With an emphasis on individual components, using HPLC-PDA techniques. Additionally, the majority of the methodologies reported on the measurement of just one or two components in polyherbal formulations. In order to achieve the greatest recovery of all the markers, the HPLC methods presented on the simultaneous quantification of multi-component traditional formulation do not provide a suitable extraction approach. As a result of the targeted marker components interfering with other sample moieties when such samples are directly injected into HPLC, lower amounts of the chemicals cannot be detected.

The study's objectives are to develop a quick, accurate, and repeatable method for identifying multiple active ingredients simultaneously in these widely used traditional medicines for quality control, as well as to suggest an improved extraction method to increase the recovery of all nine marker constituents from polyherbal Unani formulations.

**Materials and methods:**

**Samples, chemicals, solvents and standards**

Three batches of Itrifal-e-Aftiomoon and Itrifal-e-Badiyan were selected for analysis; batch I was made with components gathered in Delhi in the north of India, while batch II was made with ingredients bought in the south of India (Hyderabad). Both batches were made in accordance with the steps outlined in the National Formulary of Unani Medicine (NFUM), which was released by the Indian government. Batch III came from the Central Council for Research in Unani Medicines as a gift sample (CCRUM, Hyderabad). A university botanist identified all of the formulation's active ingredients, and a laboratory pharmacognosist verified their authenticity. All medication and formulation voucher specimens have been deposited in the university's Bioactive Natural Product Laboratory. Acetonitrile and methanol were of HPLC quality and came from Merck Specialities Private Ltd. in Mumbai. Ortho-phosphoric acid (H3PO4) 88% was purchased from Spectrochem Pvt. Ltd. Throughout the experiment, Milli Q water that had been purified using a Millipore system was used.

Sami Labs Ltd., Bangalore, provided gift samples of gallic acid 97%, ellagic acid 97%, and tannic acid 95% standards (India). Reference standards for ascorbic acid (99%), rutin (98%), and quercetin (96%), were purchased from Sigma Aldrich (USA). Standards of Sennoside A (94%), Sennoside B (89%), and Chebulinic Acid (95%) were purchased from ChromaDex in India (Bangalore). Fig. 1 provided the chemical compositions of all nine components.

**Instrumentation and conditions for chromatography**

The analysis was performed utilising a photo diode array detector (Waters 2998) with an autosampler and column oven on a Waters Alliance e2695 separating module (Waters Co., MA, USA). Empower software, which was installed with the equipment for data collection and acquisition, was used to control the device. On a C18 reverse phase, compounds were separated (RP).

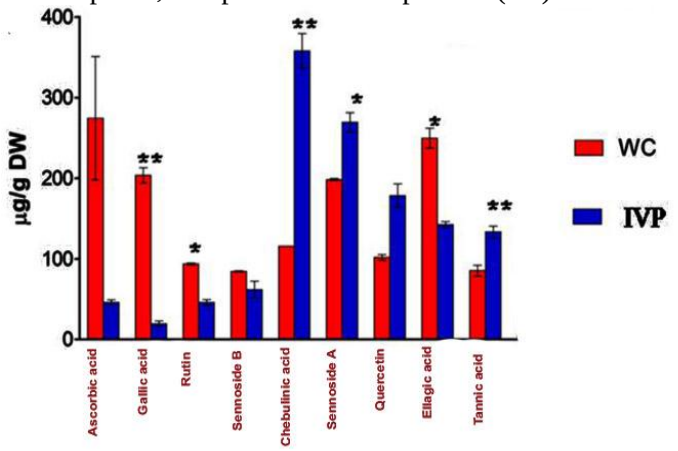



**Fig 4. Accuracy study of phenolics, flavonoids and anthraquinones.**

The solvent A (0.05%, v/v solution of ortho-phosphoric acid) and solvent B (acetonitrile containing 0.5% mobile phase A) used in the mobile phase had the following elution profiles:

0–20% B at 0–10 min, 20–22% B at 15–20 min, 22–30% B at 20–25 min, 40–50% B at 25–30 min, 50–60% B at 35–60 min, and the re-equilibration duration for each gradient elution was 15 min. A flow rate of 1.0 mL/min was used, and the column was kept at room temperature.

**Preparing a sample**

The extraction process was optimised before sample preparation for full recovery of all desired nine components of analysis using various methanol:water ratios and variable times for sonication, which were observed using the proposed HPLC method. IA and IB samples weighing one gramme each (in triplicate) were taken from each batch and put in a 50 mL conical flask with 25 mL of solvent (70% methanol) before being thoroughly mixed. It was filtered through Whatman filter paper no. 4 after being sonicated for 40 minutes at room temperature (the extraction solvent and time of sonication was optimised and selected after several trials for maximum recovery of all nine components).

To get rid of unwanted nonpolar chemicals, the filtrate was transferred to a separating funnel with a 100 mL capacity and extracted with 25 mL of hexane. Aqueous methanol extract was dried using rotavapor at a temperature below 40 °C, and the resulting residues were reconstituted in 10 mL of methanol:water (1:1, v/v) solvent before being transferred to a 25 mL volumetric flask to achieve a concentration of 40 mg/mL. Before injecting, all sample solutions were filtered with a 0.22 mm syringe filter.

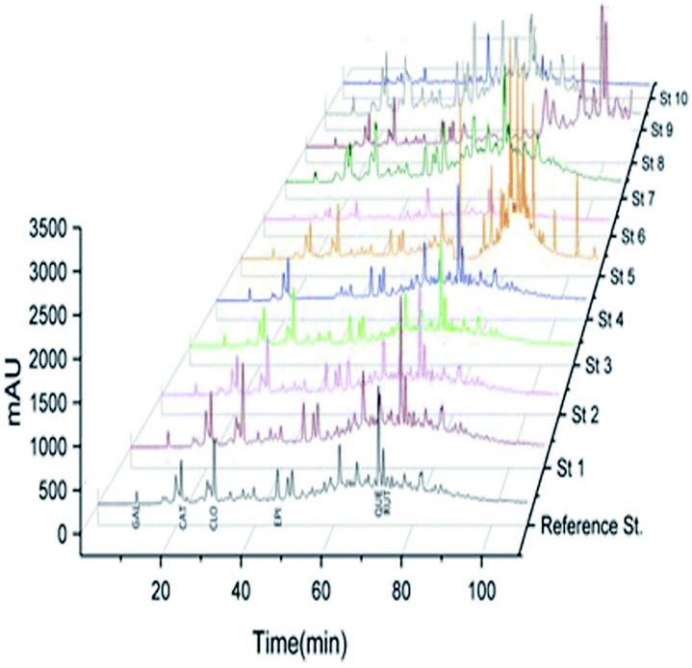



Fig 3. Intra and inter-day variations of the method

**Results and Discussion:**

Chromatographic conditions being improved Gradient elution were used to separate the majority of the formulation's components because the polarity range of the components is very constrained. The mobile phase was chosen using various ratios of methanol-water (50:50 and 80:20) and acetonitrile-water (20:80 and 60:40) with certain modifiers, such as orthophosphoric acid, formic acid, acetic acid, phosphate buffer, and acetate buffer, and tested under various gradient elution techniques. The representative HPLC-PDA chromatograms of all nine reference compounds and excellent separation of all components were achieved after numerous attempts using solvent A (0.05%, v/v solution of orthophosphoric acid) and solvent B (acetonitrile with 0.5% mobile phase A).

The UV spectra of each analyte were independently determined to obtain the maximum concentrations of each of the nine components: quercetin, ellagic acid, gallic acid, rutin, sennoside A, chebulinic acid, sennoside B, and vitamin C at 243, 271, 255, 267, 278, 269, 255, and 273 nm, respectively. 254 nm wavelength was chosen as the detecting wavelength for the analysis in order to effectively identify all nine components.

Extraction method optimization for sample preparation

To achieve satisfactory extraction efficiency, which must permit complete extraction of the target compounds while avoiding chemical alteration, the optimization of extraction process needs to be investigated?

Different solvent ratios were explored for extraction in an effort to determine the best solvent composition and extraction time. The HPLC method was also used to track the recoveries of all nine components over time. According to reports, aqueous methanol is an effective solvent for phenolic and flavonoid compound extraction. Studies on the solubility of phenolic and flavonoid compounds revealed that the best solvent was methanol-water (70:30). The goal of the extraction process optimization was to maximize the recovery of each of the nine components. In this investigation, it was discovered that the best conditions for extracting all of the components involved 70% methanol in water as a solvent and a 40-minute sonication extraction period.

In order to extract the poly herbal formulations, the best extraction time for the samples was established as 40 min in 70% methanol in water.

**Technique Verification**

According to ICH criteria, the suggested method was verified for a variety of factors, including linearity,

accuracy, and precision, limits of detection (LOD) and limits of quantification (LOQ), as well as resilience similar to the methods published by laboratories.

Limits of detection, calibration curves, and quantification Vitamin C, gallic acid, rutin, sennoside B, chebulinic acid, sennoside A, quercetin, ellagic acid, and tannic acid were among the nine reference standards dissolved in water:methanol to create standard stock solutions at 500 mg/mL concentrations (1:1).

The working standard solutions were then diluted from the stock solutions to various concentrations and kept at 4°C until they were put to the HPLC after being filtered through a 0.22 mm syringe filter and then injected for the creation of calibration plots.

For ascorbic acid, gallic acid, rutin, sennoside B, chebulinic acid, sennoside A, quercetin, ellagic acid, and tannic acid, respectively, the calibration curves were plotted with at least six suitable concentrations in triplicate in the ranges of 6.4- 320, 1.1-474, 1.8-257, 10-350, 10.6-355.8, 10-220, 5-346.8, 2.3-456.

The nine reference compounds' lowest diluted solutions were further diluted to anations of LOD and LOQ for the calibration curves. Under the current chromatographic circumstances, the LOD and LOQ were established at signal-to-noise (S/N) ratios of 3 and 10, respectively. Table 1 displays calibration curves, a regression equation, as well as LOD and LOQ for each drug.

**Specificity**

The absence of any endogenous interference at retention times of interest peaks, which is self-indicative by comparing retention times, spectra, and peak purity using PDA, served as a confirmation of the specificity.

**Accuracy and precision**

By performing the intra-day and inter-day variation tests, the precision of the procedure was ascertained. Three distinct concentrations of the standard were prepared and applied in duplicate six times each day for intra-day precisions, and similarly on six different days, for inter-day precisions. Relative Standard Deviation (RSD) was used as an accuracy indicator (Table 2).

The pre-analyzed samples were spiked with standard at three distinct known concentration levels, namely 50, 100, and 150%, and the mixes were re-analyzed using the suggested approach in order to assess the accuracy. The analysed targets' average recoveries ranged from 94.36% to 108.27%, and the RSD values were all less than 3%. (n 5 3). Data collected revealed that the procedure was accurate and dependable.

**Sample evaluation**

Itrifal-e-Aftimoon and Itrifal-e-Badiyan, two traditional poly herbal formulations that are frequently prescribed in the Unani System of Medicine, were examined in three different batches using the HPLC-PDA method, which was created and successfully used for the identification and quantification of nine marker components (USM). The optimised extraction method mentioned earlier in the section was used to analyse each sample. By comparing the retention and PDA spectra of each component, it was possible to identify the peaks in the sample chromatograms. The matching calibration curve was used to determine the content of each analyte. Figure 4 lists the markers (n 5 3) that were quantified in each formulation across three separate batches.

Rutin and sennosides were missing from the Itrifal-e-Badiyan samples, whereas all nine marker components were present in the Itrifal-e-Afti moon samples. In the three batches that were examined for both samples, there were no notable variations in the amounts of any of these components. In recent years, there has been a global increase in demand for the use of traditional medicine. There are numerous papers on the standardisation of multi-herbal formulations, where the HPLC method was used to determine the bioactive components [21]. In light of this, the RP-HPLC method described here offers a straightforward, precise, and quick method for simultaneously determining four phenolics (gallic acid, ellagic acid, chebulinic acid, and tannic acid), two flavonoids (rutin and quercetin), two anthraquinones (sennoside A and B), and one oxygenated hydrocarbon (vitamin C) in itrifals.

The simultaneous determination of these nine indicators in a conventional Unanipolyherbal formulation is the subject of this first report. The ease of the extraction process and the simultaneous identification and quantification of all nine of these bioactive components in a single chromatographic run are the method's key benefits. The quantification limits were discovered to be sufficiently low for the method to be successfully used in a variety of polyherbal formulations that contain extremely small amounts of these components. Recovery experiments were used to evaluate the approach's effectiveness, and the findings were found to be encouraging. This suggests that the procedure can be applied repeatedly with high accuracy and precision to a variety of multi-component herbal formulations. The approach can be used for samples that comprise these

components in a variety of amounts, according to the linearity studies that were done.

**Conclusion**

As a result, the suggested approach is beneficial as a dependable, quick, and efficient instrument for the quality control and standardization of various formulations, particularly traditional polyherbal formulations like Itrifals and Triphala of the Unani and Ayurvedic Systems of medicines. This straight forward multi-component assay approach will be useful for pharmacological, biopharmaceutical, and pharmaco-kinetic studies in addition to quality control of a broad number of conventional formulations.

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