RESEARCH ARTICLE

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Gallic Acid, Scopoletin, Umbelliferone, and Imperatorin in Bael avaleha

Ganesh B. Nigade[1*], Meenakshi N. Deodhar[2], Rajashree S.Chavan[1]

[1]*PDEA's Seth Govind Raghunath Sable College of Pharmacy, Saswad, Pune, Maharashtra, India.*
[2]*TMV's Lokmanya Tilak Institute of Pharmaceutical Sciences, Pune, Maharashtra, India.*

Received: 19th March, 2023; Revised: 02nd April, 2023; Accepted: 13th August, 2023; Available Online: 25th September, 2023

ABSTRACT

Bael avaleha is a semisolid ayurvedic formulation containing the fruit of bael, which acts as an anthelmintic to treat diarrhea, dysentery, and IBS. It strengthens the gastrointestinal tract, heals ulcers, and alleviates abdominal pain. The avleha contains excess sugar, which can result in faster absorption of active ingredients in the biological system. Researchers have begun scrutinizing ayurvedic formulations for quality and consistency, with quality testing being conducted to ensure that the quality of ayurvedic formulations adheres to ayurvedic standards. Phytochemical fingerprinting by spectroscopic and chromatographic techniques is becoming more popular in assessing the efficacy of ayurvedic products. Previous studies evaluated phenolic compounds, flavonoids, monoterpenes, and sesquiterpenes in Bael fruit using Gas Chromatography Mass Spectrometry (GC-MS), HPTLC, and HPLC methods.This study evaluated marketed bael avaleha formulations (BA I and II) for organoleptic characteristics, physical parameters, and phytoconstituents. A UV-vis spectrophotometric study was conducted for quantitative analysis of phytochemicals. The extract of bael avaleha formulations BA I and BA II were found to have a total phenolic content of 42.34 ± 0.090 mg GAE/g and 38.52 ± 0.065 mg GAE/g, a flavonoid content of 14.34 ± 0.070 mg QE/g and 12.42 ± 0.086 mg QE/g, sugar content of 69.44 ± 0.020 mg Glu/g and 67.18 ± 0.065 mg Glu/g, and a reducing sugar content of 31.34 ± 0.025 mg Glu/g and 29.08 ± 0.047 mg Glu/g, respectively. The Fourier transform infrared (FTIR) spectra of extract of bael avaleha formulations were recorded in region 4000–400 cm^{-1} and confirmed the presence of hydroxy group, aromatic C-H stretch, C=C and C-O group. RP-HPLC method was developed and used for the estimation of gallic acid, scopoletin, umbelliferone, and imperatorin in the extract of Bael avaleha formulations.

Keywords: Bael avaleha, Ayurveda, Quantitative phytochemical evaluation, UV-vis spectroscopy, FTIR, HPLC.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.3.29

How to cite this article: Nigade GB, Deodhar MN, Chavan RS. Development and Validation of RP-HPLC Method for Simultaneous Estimation of Gallic Acid, Scopoletin, Umbelliferone, and Imperatorin in Bael avaleha. International Journal of Pharmaceutical Quality Assurance. 2023;14(3):627-636.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The world has diverse traditional systems of medicine, such as those originating in Africa, China, Korea, and India.[1] Traditional medicine systems often use of medicines obtained from natural sources such as plants, animals, and minerals.[2,3] The majority population of developing and advanced countries relies on traditional and alternative medicines for primary healthcare.[4,5] Traditional medicine systems work on the basis of knowledge, practices, and experiences.[6,7] Ayurveda and Siddha are well-recognized ancient and indigenous Indian traditional medicine systems.

Ayurveda is the oldest and the most widely practiced traditional medicine system.[8] It considers all dimensions of wellness, encompassing an individual's psychological, physiological, and social components.[9] Different formulations used in *ayurveda* often exhibit diverse therapeutic effects.[10-12] In *ayurveda*, various types of dosage forms, including Vati, Churna, Kwatha, Asava, Arishta, Kalka, Avaleha, etc. are available in the market. Ayurvedic formulations are prepared by traditional methods using several herbs and minerals.[13,14]

Bael Avleha is one of the semisolid ayurvedic formulations that contain bael fruit (*Aegle marmelos (L)*). It acts as an anthelmintic help to treat diarrhea, dysentery, and IBS. It strengthens G.I. tract, helps to heal the ulcers in the gastrointestinal tract, and alleviates abdominal pain.[15-17] Avleha contains excess sugar, and sugar is known to be absorbed more rapidly than other nutrients. This could result in faster absorption of the active ingredients in the biological system. The principle phytoconstituents of Bael fruit include alkaloids like aegeline and marmelline [18,19,] coumarins like umbelliferone

**Author for Correspondence: ganeshnigade2984@gmail.com*

and scopoletin and furanocoumarins like marmelosin, marmesinin, imperatorin and angelicin[20,21,] flavonoids like rutin, quercetin and various vitamins like ascorbic acid, niacin, riboflavin, and thiamin, minerals like phosphorus and calcium.[17] Phytochemicals in bael fruits have several therapeutic uses such as antibacterial,[22-24] antidiarrhoeal,[25-27] antiviral,[28,29] antioxidant,[30] radioprotective effects,[31] cytotoxic effect,[32] antineoplastic effects,[33] chemopreventive,[34] gastro duodenal protective and antiulcerogenic properties,[35,36] antigenotoxic activity,[37] diuretic activity,[38] Antifertility activity,[39,40] antimutagenic activity.[41]

In view of the various phytochemicals and therapeutic uses of bael avleha becomes the important formulation of ayurveda. Researchers have begun scrutinizing Ayurvedic formulations more closely for quality and consistency due to the need for higher-quality Ayurvedic products.[42-44] Ayurvedic industries accomplish quality testing in order to ensure that the quality of Ayurvedic formulation adheres to ayurvedic standards.[45] The effectiveness of ayurvedic formulations is directly related to the phytochemical profile. Thus, understanding it is crucial.[46] Some of the steps involved in determining an ayurvedic product's phytochemical profile include a preliminary phytochemical examination, a phytochemical fingerprint, and marker-based quantification.[47] The use of phytochemical fingerprinting to assess the efficacy of ayurvedic products is becoming more popular. Analyzing phytochemicals is facilitated by marker-based fingerprint profiling of traditional herbal remedies by spectroscopic and chromatographic techniques.[48–50]

In previous work, Charoensiddhi *et al. reported the GC-MS method for estimating* ascorbic acid, total carotenoids, monoterpenes and sesquiterpenes in bael fruit.[51] Dhalwal *et al.,* simultaneous quantified umbelliferone and psoralen in fruit pulp of bael by HPTLC.[52] Shailajan *et al.,* have reported the HPTLC method for estimating marmelosin from bael fruit pulp methanol extract.[53] Yadav *et al.,* estimated tannin, vitamin B2, vitamin C, and organic acids as oxalic, tartaric and malic acid and sugars in Bael fruit by HPLC.[54] Dhan *et al.,* determined different phenolic acids and quercetin in Bael fruit by LC-MS method.[55] Bhattacherjee *et al. estimated* marmelosin and psoralen in Bael fruit by HPLC method.[56] Shinde *et al.,* simultaneously estimated umbelliferone, marmelosin and scopoletin from Bael fruit by RP- HPLC method.[57] Singh *et al.,* developed fingerprints of polyherbal marketed formulations of *Aegle marmelos* by TLC and HPLC.[58] Shelke *et al.* determined marmelosin from bael fruit by HPLC method.[59] Considering the phytochemicals and therapeutic importance of bael fruit, we developed simultaneous estimation of gallic acid, scopoletin, umbelliferone and imperatorin by HPLC from Bael avaleha.

MATERIALS AND METHOD

Instrument

Ragatech microwave was used for the extraction; a Jasco V-630 spectrophotometer and Shimadzu FTIR 8400S spectrophotometer were used for UV-vis data and FTIR spectral data, respectively. The chromatogram was recorded by using high-performance liquid chromatography LC-4000 (Jasco) system.

Reagents and Chemicals

Bael avaleha of Nagarjuna (BA I) and Sandu (BA II) were procured from a local pharmacy. Merck (India) limited gave us HPLC-grade methanol and water. All of the markers and solvents used were of scientific grade and bought from Merck India Ltd., SD Fine Chemicals Ltd., and Yucca Enterprises, all of which are based in Mumbai, India.

Preparation of Stock Solution

The stock solutions of gallic acid, scopoletin, umbelliferone, and imperatorin (100 µg/mL) were prepared by using methanol (AR grade).

Sample Preparation

The Bael avaleha formulations (1g) were subjected to extraction using 20 mL of methanol in a microwave at a power of 280W for duration of 10 minutes. A rotavap concentrated the extracts and the residue was preserved in an airtight container for further analysis.

Experimental

Organoleptic evaluation

The organoleptic evaluation of Bael avaleha formulations were conducted for the characteristics of odor, color, appearance and taste.

Physical parameter evaluation

Physical properties of Bael avaleha formulations were analyzed using ayurvedic pharmacopoeial methods.[60]

Qualitative phytochemical evaluation

Qualitative phytochemical evaluations of Bael avaleha formulations were undertaken by different phytochemicals tests viz. the alkaline reagent test, Benedict's test, Libermann-Burchard test, Salkowski test etc.[61,62]

Quantitative Photochemical Analysis

Total phenolic content

The Folin Ciocalteu method was applied to determine the phenolic content of Bael avaleha formulations.[5,63] A linear regression analysis was performed using the gallic acid standard calibration curve. Bael avaleha extracts were diluted with distilled water (1:15) to create a test solution (100 mg/mL). 500 L of Folin-Ciocalteu reagent (2 N) was incorporated to a test solution. After waiting for five minutes, 2 mL of washing soda (20%) was added to the volumetric flask. After an hour of incubation, a UV-visible spectrophotometer was used to record the absorption of the resulting solution at 765 nm.

Total flavonoid content

The aluminum chloride method determined the total quantity of flavonoids in Bael avaleha formulations.[64] The quercetin standard calibration curve was used for a linear regression analysis. A stock solution of Bael avaleha extract (1-mL)

and 10% aluminium chloride (0.1 mL) were combined in a volumetric flask. In a mixture, 0.1 mL of 1M potassium acetate was incorporated and kept for 1 to 2 minutes. Final 5 mL volume made up with distilled water. After 30 minutes, absorption at 430 nm was measured using UV-vis spectrophotometer.

Total sugar content

The total quantity of sugar content in Bael avaleha formulations were estimated by the phenol-sulfuric acid method.[65] The D-glucose standard calibration curve was used for linear regression analysis. In a volumetric flask, 1-mL of stock solution (100 mg/mL) of extract of Bael avaleha, 1mL of 5% phenol, and vortexed after the addition of 5 mL of concentrated sulfuric acid to the mixture. After 30 minutes of incubation, a UV-visible spectrophotometer was used to record the absorption of the resulting solution at 540 nm.

Total reducing sugar

The total quantity of reducing sugar in Bael avaleha formulations was estimated by DNS reagent method.[66] The D-glucose standard calibration curve was used for a linear regression analysis. A test solution of extracts of Bael avaleha (100 mg/mL) was produced by diluting it with distilled water (1:3). A total of 3 mL of DNS reagent were combined and heated in water for five minutes. The absorption was recorded in a UV-vis spectrophotometer at 540 nm.

UV-vis spectrophotometric study

UV-vis absorption spectra of extract of Bael avaleha formulations were recorded using a Jasco UV-vis spectrophotometer in the spectral range of 200–400 nm.

FTIR study

The FTIR spectrum of the extract of Bael avaleha formulations was obtained using the diffuse reflectance spectroscopy (DRS) technique. The spectrum was measured within the frequency range of 4000–400 cm^{-1}.

HPLC Analysis

The chromatographic investigation was conducted using the HPLC with PDA detector (Jasco-MD 4000). The column used was Jasco Finepak SIL C18 T-5 column (250 × 4.6 mm, 5 µm). An isocratic mobile phase consisting of methanol and water with 0.1% acetic acid (60:40% v/v) was passed through the system for 20 minutes at a 0.7 mL/min flow rate. The sample injection volume was 10 µL. The mobile phase was degassed and filtered using a 0.4 µm membrane filter prior to use. HPLC analysis was conducted at a column temperature of 30°C. The resulting data was analyzed using Chrom NAV 2.0 software.

System Suitability and Method Validation

By examining six replicates of the marker solution, the system suitability of the method was evaluated. The study focuses on the analysis of variables like the tailing factor (T), theoretical plate number (N), and resolution (Rs).

Guidelines from the ICH were used to prove that the suggested HPLC method works.

Specificity

Specificity is the ability to identify the chemical correctly, even if there are other things that could get in the way. The study ensured specificity by comparing the chromatograms of the extract sample, with the marker solution serving as the reference. The retention time of each marker was examined to assess the peak purity.

Linearity

Linearity is the ability of an analysis method to give test results that are directly related to the concentration of analyte in the sample. In the HPLC system, working solutions of gallic acid (2–10 µg/mL), umbelliferone (2–10 µg/mL), scopoletin (2–10 µg/mL), and imperatorin (10–30 µg/mL) were separately injected. The peak area v/s concentration of the applied markers was plotted to obtain a straight line, slope, and correlation coefficient (R2) equation.

Detection and quantitation limit

The level of detection (LoD) signifies the smallest detectable amount of analyte in a sample, while limit of quantitation (LoQ) represents the minimum quantity of analyte that can be accurately and precisely measured. Detection and quantitation limit was determined using slope of the corresponding calibration curve as well as standard deviation (SD) of peak regions of each marker.

Precision

Repeated measurements of the same homogenous samples taken under controlled conditions were used to determine the precision of an analytical technique. This can be done by evaluating intraday and intermediate precision (repeatability). Intraday precision was assessed for three different concentrations of each marker by applying three replicates of each concentration to an HPLC system. The inter-day precision was evaluated for three distinct concentrations of each marker by implementing three replicates of each concentration on different days.

Robustness

The ability of an analytical procedure to withstand intentional variations in method parameters without being significantly affected is known as robustness. The small but deliberate variations in the column temperature (30 ± 2°C) and flow rate (0.7 ± 0.1 mL/min) of the optimized HPLC method were done to determine the robustness and findings were expressed in %RSD.

Accuracy

The accuracy of an analytical procedure is determined by how closely the value obtained aligns with the accepted or reference value. The reliability of the procedure was evaluated using the conventional addition technique. The extracts of Bael avaleha formulations were spiked with a known amount of markers, and the percentage recovery was calculated.

Statistical Analysis

All statistical data were calculated using Microsoft Excel.

RESULT AND DISCUSSION

Organoleptic Evaluation

The marketed Bael avaleha formulations (BA I and II) were evaluated for organoleptic characteristics. Both Bael avaleha formulations were semisolid, having brown in color sour and astringent taste. As per ayurvedic pharmacopeia, Bael avaleha formulations were identified and confirmed by the organoleptic evaluation and data reported in Table 1.

Physical Evaluation

Physical parameters of marketed Bael avaleha formulations (BA I and BA II) were evaluated. Foreign matter was not found in both Bael avaleha formulations, indicating its purity. The total ash value of Bael avaleha formulations was within the limit, indicating that inorganic residue was less and confirmed the quality of formulations.[67-69] Both Bael avaleha had acid-insoluble ash values below 2%, indicating that a limited quantity of the inorganic component was acid-soluble, which confirmed that the Bael avaleha formulations was not adulterated.[67–69] Phytoconstituents like sugars and mucilage, etc., were evaluated using a water-soluble extractive value and tannins, resins, and alkaloids were evaluated using an alcohol-soluble extractive value and outcomes were reported in Table 2.

Qualitative Phytochemical Evaluation

The qualitative phytochemical analysis of the marketed Bael avaleha formulations (BA I and II) was conducted, and the results are given in Table 3. These qualitative tests rely on color or precipitation reactions to indicate the presence of distinct chemical compounds.[70] It revealed that the extracts of Bael avaleha formulations contained alkaloids, flavonoids, phenol, tannins, coumarins and carbohydrates.

Phytochemical Evaluation

Compounds isolated from nature have been shown to have therapeutic potential as an antioxidant and anti-inflammatory effects in the intestines, among their many therapeutic uses. Secondary metabolites that have been demonstrated to have anti-inflammatory actions in the intestines include phenolic compounds, flavonoids, alkaloids, coumarins, curcuminoids, tannins, and terpenes.[71] Phenolic compounds and flavonoids are naturally occurring and exhibit potential as therapeutic agents for various disorders, including neurological diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases, antibacterial and antiviral activities, as well as antimutagenic effects and aging-related conditions.[72–75] The extracts of Bael avaleha formulations (BA I and II) were evaluated for phytochemicals by UV-vis spectrophotometer using the standard curve and shown in Table 4. It found that Bael avaleha formulations are a rich source of phenolic compounds. Flavonoids were measured depending on the development of complexes between flavonoids and aluminum.[76] Total sugar determined by formation phenol furfural derivatives.[77] On reducing the 3,5-dinitrosalicylic acid reagent and sugar that has been converted to sugar acid in an alkaline solution, the reducing sugars were determined.[78]

The Bael avaleha formulations (BA I and II) were subjected to UV radiation in the 200–400 nm region. Extracts of Bael avaleha formulations (BA I and II) showed absorbance peaks in the UV-vis spectra (Figure 1). Bael avaleha formulation I (BA I) showed peaks at 231 and 273.5 nm and Bael avaleha formulation II (BA II) showed a peak at 222 and 275.5 nm. The UV-vis spectra of each Bael avaleha formulation showed two band absorption spectra due to aromatic rings confirming hydroxy coumarins and phenolic compounds.[79,80]

Table 1: Organoleptic evaluation

Sr. no.	*Particular*	*BA I*	*BA II*
1	Appearance	Semisolid	Semisolid
2	Color	Brown	Brown
3	Odor	Sour	Sour
4	Taste	Sour and astringent	Sour and astringent

Table 2: Data of physical evaluation

Sr. no.	*Parameters*	*BA I*	*BA II*
1	Foreign matter	Nil	Nil
2	Moisture content	4.70	5.30
3	Total ash	0.85	0.92
4	Acid insoluble ash	0.27	0.35
5	Alcohol soluble extractive value	83.10	85.50
6	Water soluble extractive value	37.10	41.50

FTIR Study

The solid-state infrared (IR) spectra (Figure 2) indicate that the extracts of Bael avaleha formulations I and II exhibit a broad peak in the 3480 to 3410 cm^{-1} range related to the hydroxy group. An observed peak in the 3100–3050 cm^{-1} range also relates to the aromatic (C-H) stretch. The stretching at 2900 to 2950 cm^{-1} confirmed the aliphatic (C-H) and 1645 to 1600 cm^{-1} confirmed the aromatic C=C group. The medium appearance of the vibration at 1455-1440cm^{-1} confirmed C-H bending. The existence of the C-O group was shown by stretching between 1065 and 1050 cm^{-1}. C=C bending was verified by peak at 885 to 860 cm^{-1}.[81–83]

HPLC Analysis

The simultaneous quantification of gallic acid, scopoletin, umbelliferone, and imperatorin in Bael Avaleha formulations has been made easier and quicker with the development of a simple and rapid HPLC-PDA method. The process of optimizing simultaneous chromatographic separation conditions involved making deliberate changes to one parameter at a time, while keeping all other variables unchanged. The process required making adjustments to various factors, including mobile phase, stationary phase, flow rate, and detection wavelength. Four phytochemicals of interest were effectively separated using an isocratic elution of methanol: water contain 0.1% acetic acid (60:40) with flow rate 0.7 mL/min, 20 min run time and using C 18 column. The optimization of the chromatographic separation conditions, considering factors such as peak shape, symmetry, and resolution. The chromatograms of the extracts

Table 3: Data of qualitative phytochemical evaluation

Sr. No.	*Phytochemical*	*Test*	*Observation*	*BA I*	*BA II*
1.	Alkaloids	Picric acid	Yellow color	Positive	Positive
		Dragendorff's	Orange red precipitate	Positive	Positive
2.	Flavonoids	Alkaline reagent	Intense yellow color	Positive	Positive
		Shinoda	Deep pink color	Positive	Positive
		Mayer's	Yellowish precipitate	Positive	Positive
3.	Phenols	$FeCl_3$	Yellowish orange color	Positive	Positive
4.	Tannin	Lead sub-acetate	Gelatinous precipitate	Positive	Positive
5.	Coumarins	Sodium hydroxide solution	Dark yellow color	Positive	Positive
6.	Carbohydrate	Benedict's	Orange color	Positive	Positive
		Fehling's	Brick-red precipitate	Positive	Positive
		Molisch's	Purple color	Positive	Positive
7.	Steroids	Libermann-burchard	No bluish green color	Negative	Negative
8.	Terpenoids	Salkowski	No intense red-brown color	Negative	Negative
9.	Saponins	Froth formation	No froth formation	Negative	Negative

Table 4: Data of phytochemical evaluation

Sr. No.	*Parameters*	*BA I*	*BA II*
1	Total phenolic	42.34 ± 0.090 mg GAE/g	38.52 ± 0.065 mg GAE/g
2	Total flavonoid	14.34 ± 0.070 mg QE/g	12.42 ± 0.086 mg QE/g
3	Total sugar	69.44 ± 0.020 mg Glu/g	67.18 ± 0.065 mg Glu/g
4	Total reducing sugar	31.34 ± 0.025 mg Glu/g	29.08 ± 0.047 mg Glu/g

UV-vis Spectrophotometric study

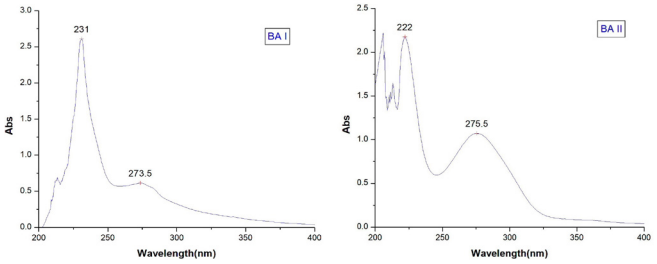

Figure 1: UV-vis spectrum of a) BA I, b) BAII

as shown in Figure 3, indicate that the four phytochemicals were successfully separated within a time frame of 20 minutes. The chromatographic peaks observed in the marketed Bael avaleha formulations showed a correlation with the markers. The extracts of Bael Avaleha formulations were spiked with the markers to assist in peak identification confirmation. The RT for gallic acid, scopoletin, umbelliferone, and imperatorin were 2.95, 4.19, 6.9, and 11.71 minutes respectively.

System Suitability and Method Validation

System suitability tests are crucial for ensuring the reproducibility of any chromatographic system. These tests

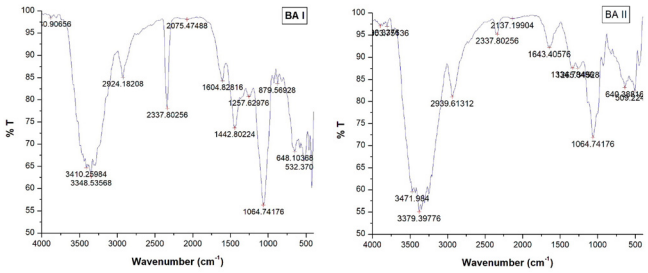

Figure 2: FTIR spectrum of extract of a) BA I, b) BA II

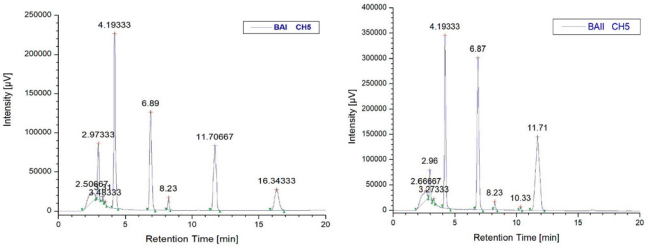

Figure 3: Chromatogram of a) BA I b) chromatogram of BA II

play a vital role in the chromatographic method. The HPLC method is considered suitable when the tailing factor < 2 and the theoretical plates > 2000, and the capacity factor and the results are shown in Table 5.

The proposed HPLC method was validated by assessing various parameters, as the ICH required.

Specificity

The specificity evaluation involved comparing the chromatograms obtained from the blank solution, marker solution, and extract. The peaks for gallic acid, scopoletin, umbelliferone, and imperatorin in the extracts of Bael avaleha formulations were confirmed by correlating the retention time and peak purity with that of the markers.

Table 5: Data of system suitability test

Marker	*Number of theoretical plates (N)*	*Tailing factor (T)*	*Resolution (Rs)*
Gallic acid	4548	1.019	10.37
Scopoletin	7956	1.176	14.26
Umbelliferone	9008	1.157	17.11
Imperatorin	11081	0.894	N/A

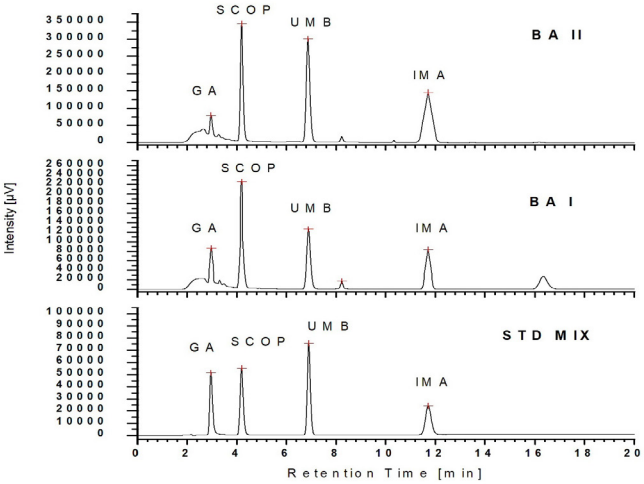

Figure 4: Stack view of chromatogram of standard mixture, beal avleha i and beal avleha II

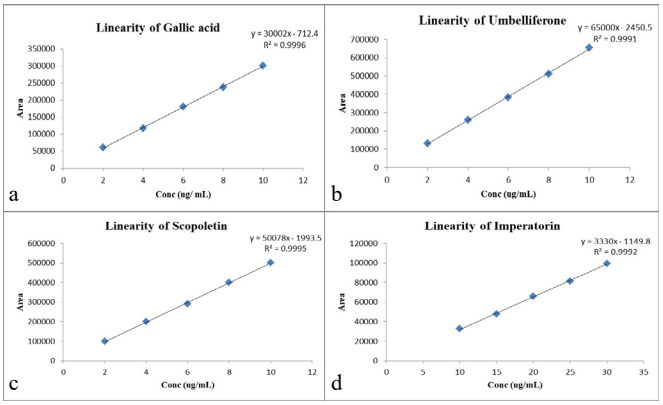

Figure 5: a) Linearity of gallic acid b) Linearity of umbelliferone c) Linearity of scopoletin d) Linearity of imperatorin

Linearity

Within the context of linearity, the linear relation between the marker concentrations and their associated peak area responses was determined. For gallic acid, umbelliferone and scopoletin, linear correlation was achieved at a range of concentrations of 2–10 µg/mL; for imperatorin, it was 10–30 µg/mL. It has been noted that the peak area is directly proportional to the concentration ($R^2 = 0.999$) of each marker. All statistical data is shown in Table 6.

Detection and Quantitation Limit

Detection and quantitation limits for markers were determined using SD of the intercept and slope of a calibration curve.

Table 6: Data of linear, regression, detection and quantitation limit

Marker	*RT*	*Linearity range (*ug/ ML*)*	*Equation*	*R^2*	*Detection limit*	*Quantitation limit*
Gallic acid	2.96 ± 0.013	2-10	y = 30002x–712.4	0.9996	0.55	1.68
Scopoletin	4.19 ± 0.011	2-10	y = 50078x–1993.5	0.9995	0.65	1.97
Umbelliferone	6.9 ± 0.014	2-10	y = 65000x–2450.5	0.9991	0.65	1.98
Imperatorin	11.71 ± 0.021	10-30	y = 3330x–1149.8	0.9992	2.54	7.72

Table 7: Data of precision

Parameters/ Markers	*Intra-day*				*Inter-day*		
	*Concentration (*µg/mL*)*	*Mean area (n=3)*	*SD*	*%RSD*	*Mean area (n = 3)*	*SD*	*%RSD*
	4	117406	369.43	0.3146	117423	484.17	0.4123
Gallic acid	6	181041	690.20	0.3812	181112	773.76	0.4272
	8	237671	569.01	0.2394	236980	966.79	0.4079
	4	199078	827.83	0.4158	198735	697.57	0.3510
Scopoletin	6	291057	896.01	0.3078	291192	862.14	0.2960
	8	401081	905.70	0.2258	401647	1090.96	0.2716
	4	259366	504.54	0.1945	259001	822.98	0.3177
mbelliferone	6	380937	881.15	0.2313	381331	1057.12	0.2772
	8	512996	3633.98	0.7083	512638	3121.40	0.6088
	15	47369	372.73	0.7868	47276	260.96	0.5519
Imperatorin	20	65521	401.77	0.6131	65480	432.39	0.6603
	25	81575	346.04	0.424	81423	337.18	0.4141

Table 8: Data of robustness

Parameters/Markers	*Conc. (ug/mL)*	*Column temp. (°C)*	*Mean area*	*%RSD*	*Flow rate (mL/min)*	*Mean Area*	*%RSD*
	6	28	179719	1.053	0.6	179117	1.167
Gallic acid	6	30	180342	0.4651	0.7	180342	0.4651
	6	32	180943	1.117	0.8	181015	1.211
	6	28	291171	0.9713	0.6	290323	1.014
Scopoletin	6	30	291935	0.4253	0.7	291935	0.4253
	6	32	292231	1.109	0.8	291113	1.124
	6	28	381107	0.985	0.6	380657	1.153
Umbelliferone	6	30	381765	0.4371	0.7	381765	0.4371
	6	32	382234	1.121	0.8	382341	1.191
	20	28	65432	1.013	0.6	65011	1.154
Imperatorin	20	30	65971	0.4246	0.7	65971	0.4246
	20	32	66239	1.127	0.8	66107	1.1967

Table 9: Data of recovery studies

Parameters/ Markers	*Amount of standard added (µg/mL)*	*% Recovery ± SD*	
		BA I	*BA II*
	4	99.91 ± 0.033	99.94 ± 0.015
Gallic acid	6	99.93 ± 0.031	99.98 ± 0.022
	8	100.15 ± 0.072	99.89 ± 0.052
	4	99.89 ± 0.029	100.12 ± 0.053
Scopoletin	6	99.92 ± 0.037	100.07 ± 0.039
	8	100.22 ± 0.047	99.98 ± 0.034
	4	100.93 ± 0.046	99.84 ± 0.041
Umbelliferone	6	100.20 ± 0.047	100.01 ± 0.073
	8	99.92 ± 0.041	100.29 ± 0.051
	10	99.90 ± 0.037	99.92 ± 0.33
Imperatorin	15	99.97 ± 0.044	99.96 ± 0.37
	20	99.94 ± 0.061	99.99 ± 0.076

Detection and quantitation limit for gallic acid, scopoletin, umbelliferone, and imperatorin are shown in Table 6.

Precision

The intra-day precision and inter-day precision for the peak area for all markers were determined by repeated assessment. The % RSD for each marker was fond to be less than 2, indicating a high degree of precision in the developed HPLC method and shown in Table 7.

Robustness

Developed HPLC method was found to be robust in terms of variations in the column temp (30 ± 2°C) and flow rate (0.7 ± 0.1 mL/min). The results obtained for robustness studies and %RSD are shown in Table 8.

Accuracy

The standard addition method was used to determine the accuracy of the developed HPLC method. The Bael avaleha formulations were analyzed by adding a known quantity of markers to the extract. The percent recovery of the markers was calculated. The results obtained were shown in Table 9.

Table 10: Data of quantification of markers

Parameters/Markers	*Quantification*	
	BA I (g/100 gm)	*BA II (g/100 gm)*
Gallic acid	0.0202 ± 0.005	0.0273 ± 0.007
Scopoletin	0.0325 ± 0.012	0.0564 ± 0.015
Umbelliferone	0.2063 ± 0.015	0.4693 ± 0.031
Imperatorin	0.1027 ± 0.010	0.1991 ± 0.013

Quantification

Developed HPLC method was used for the determination of gallic acid, scopoletin, umbelliferone, and imperatorin in extract of Bael avaleha formulations. The amount of gallic acid, scopoletin, umbelliferone, and imperatorin was calculated by linear regression, as shown in Table 10.

CONCLUSION

The current study evaluated the Bael Avaleha formulations (BA I and II) using organoleptic, physical, phytochemical, spectroscopic, and chromatographic analyses. The phytochemical content of Bael Avaleha formulations was calculated using UV-vis spectrophotometric techniques by linear regression analysis. In Bael avaleha formulations, significant quantities of flavonoids, phenolic compounds, and sugar content have been found by phytochemical evaluation. Bael avaleha formulations showed evidence of many functional groups of complex phytochemicals in their FTIR spectra. HPLC method developed for the estimation of gallic acid, scopoletin, umbelliferone, and imperatorin in Bael avaleha formulations was discovered to be simple, precise, and reliable. This method enables a comprehensive analysis of the Bael avaleha formulations.

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