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Research Article

Phytochemical Screening, Spectroscopic & HPTLC Evaluation of Amalaki Formulation

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Abstract: *Amalaki*, i.e., Indian gooseberry, is a medicinal plant with rejuvenator, anti-aging, and adaptogenic properties. It has a significant amount of ascorbic acid, an essential nutrient for various biochemical and physiological processes in the body. Amalaki is used in different ayurvedic formulations like churna, juice, rasayan, chyavanaprash, dhatri lauha, dhatryadi ghrita, and triphala churna. Even so, more work was needed to establish quality control standards for Amalaki formulations. This is because the plant is considered safe, and there are few reports of adverse effects. Therefore, there needs to be more incentive to conduct rigorous studies on the safety and efficacy of Amalaki formulations. Despite this lack of research, Amalaki is still widely used with few reported side effects, making it an attractive option for those seeking natural remedies. In the present study, the phytochemical screening & physical evaluation were carried out of *Amalaki* formulations. The study includes determining organoleptic characters, moisture contents, ash, extractive values, etc. Marketed amalaki formulations were extracted in the microwave using solvent methanol: water (70:30). UV-Vis spectrophotometer was used for quantitative phytochemical evaluation of extracts of amalaki formulations. Alkaloids, flavonoids, carbohydrates, phenols, and tannins were found in the qualitative phytochemical evaluation of amalaki formulations. Total phenolic, flavonoid, tannin, sugar, and reducing sugar content of extract of Amalaki formulation AMK I & AMK II was found to be 114.11±0.035mg GAE/g & 116.12 ± 0.020 mg GAE/g, 29.15 ± 0.0029 mg QE/g & 29.75 ± 0.0020 mg QE/g, 4.50 ± 0.0085 mg CE/g & 4.70 ± 0.0079 mg CE/g, 7.34 ± 0.0034 mg Glu/g & 7.44 ± 0.0043 mg Glu/g & 3.41 ± 0.0035 mg Glu/g & 3.65 ± 0.0031 mg Glu/g, respectively. The FT-IR spectra of the extract of amalaki formulations were recorded in the region 4000–400 cm^{-1}. They confirmed the presence of the O-H, aromatic C-H stretch, C=C & C-O groups. The rutin, ascorbic acid, gallic acid, & kaempferol concentrations in the extracts of amalaki formulation were effectively estimated using the HPTLC method for marketed amalaki formulations.

Keywords: Amalaki, Ayurveda, Quantitative Phytochemical Evaluation, UV-Vis Spectroscopy, FTIR, HPTLC

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1. INTRODUCTION

Amalaki, i.e., Indian gooseberry, is a medicinal plant botanically identified as *Phyllanthus emblica Linn.* In traditional medical practices such as Ayurveda, Siddha, Unani, and Chinese medicine, amalaki plays a vital role.[1-3] According to Ayurvedic text, amalaki has vayasya (rejuvenator, anti-aging), amrutaphala(longevity for the human being) & rasayana (adaptogenic) qualities.[4,5]Amalaki has traditionally been recognized as a tonic and its medicinal potential.[6] It treats digestive problems, relieves asthmatic conditions, and helps manage fever.[7,8]Amalaki stimulates hair growth, improves heart health, contributes to improved vision, rejuvenates the body, and heightens intellectual acuity. [3,8,9,10] The anticancer, antioxidant, hepatoprotective, hypoglycemic, and hypolipidemic properties of the amalaki fruit have been reported.[11-16] The secondary metabolites of amalaki fruit have nutrient qualities & therapeutic benefits. It contains phytochemicals such as alkaloids, amino acids, carbohydrates, flavone glycosides, flavonol glycosides, mucic acid, norsesquiterpenoids, phenolic acids, phenolic glycosides, sesquiterpenoids, and tannins.[6,10,16] It is a rich source of ascorbic acid, an essential nutrient for various biochemical and physiological processes in the body.[18,19]Amalaki fruits are used in different ayurvedic formulation like amalaki churna, amalaki juice, amalakirasayan, chyavanaprash, dhatri lauha, dhatryadi ghrita, triphala churna. In view of the commercialization of various amalaki formulations, quality control standards are becoming highly significant. It is essential to analyze amalaki formulations to ensure their quality. Quality testing is performed in Ayurvedic industries to ensure the quality of Ayurvedic formulation meets the standards set forth by Ayurveda.[15] The quality control tests mentioned in the literature of Ayurveda are based on observation. [16] The ayurvedic pharmacopoeial specifications perform the organoleptic, pharmacognostic, and quantitative evaluation of ayurvedic formulations.[17] However, only observation-based evaluation & pharmacognostic evaluation is not reliable for quality testing of the ayurvedic formulation. World Health Organization (WHO) emphasizes qualitative and quantitative analysis of phytochemicals from traditional herbal medicines.[18] The active phytochemicals in the ayurvedic formulations are responsible for accomplishing the intended therapeutic & pharmacological effects.[19-21] Investigation of the phytochemicals in the ayurvedic formulation is a valuable resource for determining the quality of the formulations.[22-24]The phytochemical profile is important as it directly impacts the efficacy of the ayurvedic formulations.[25] Phytochemical profiling of ayurvedic formulations include preliminary phytochemical investigation, phytochemical fingerprints, and marker-based quantification.[26] Phytochemical fingerprint has emerged as an effective method for evaluating the quality of ayurvedic formulation. Marker-based fingerprint profiling of traditional herbal medicines by spectroscopic & chromatographic methods is a valuable tool for analyzing phytochemicals.[27-29] Spectroscopic fingerprinting helps to generate a standard signal pattern for analyzing the phytochemicals found in traditional herbal medicines.[30,31,32] Two primary features of chromatographic fingerprinting, "sameness" and "difference," are used to analyze & compare the phytochemical of traditional herbal medicines.[33,34] Therefore, acquiring trustworthy spectroscopic and chromatographic fingerprints that accurately reflect the pharmacologically active and chemically distinctive components of Ayurvedic formulations is of the utmost importance.[35-37] Thus, evaluating phytochemicals in ayurvedic formulations for quality, safety, and effectiveness requires using several modern analytical methods. Spectroscopic and chromatographic analytical method development may be valuable techniques for identifying marker molecules in the phytoconstituents and developing quality control protocols for ayurvedic formulations. In previous work, Ghosal et al reported that Emblicanin A & B as active constituents of amalaki.[38]Shishoo et al. estimated Vitamin C content by fluorimetric method from amla powder & Chyavanprash.[39] Srinivasan et al. determined the ascorbic acid content in amala fruit by HPLC.[40]Chakraborthy et al. established the HPTLC method for determining ascorbic acid by various amla varieties.[41]Sawant et al. reported the HPTLC method for the determination of gallic acid in the powder of amala fruit.[42] Patel et al. quantified ascorbic acid & gallic acid by using separate mobile phases from amla powder, Amalant tablets by HPTLC.[43] Bansal et al. quantified vitamin C, phenolic acids, and flavonoids in amala juice by HPLC.[44]Jirge et al. estimated kaempferol, rutin, and quercetin from amla & bhumi amla formulations by HPTLC.[45] Considering the phytochemicals & therapeutic importance of amalaki, we have developed phytochemical screening, spectroscopic analysis & simultaneous estimation of ascorbic acid, gallic acid, rutin& kaempferol by HPTLC from amalaki formulation.

2. MATERIALS & METHODS

2.1. Samples

Amalaki capsules of Zandu (AMK I) and Amalaki churna of Sharangdhar (AMK II) were procured from a local pharmacy.

2.2. Equipment

Ragatech Microwave was used for the extraction; a Jasco V-630 spectrophotometer & Shimadzu FTIR 8400S spectrophotometer
were used for UV-VIS data & FTIR spectral data, respectively. The CAMAG HPTLC system, TLC scanner 3 with WINCATS software, was used for the chromatographic data.

2.3. Sample Preparation

Amalaki formulations (1g) were extracted with 10 mL solvent methanol: water (7:3) in the microwave at 280 W for 10 minutes. A rotavap concentrated the extracts, and the residue was preserved in an airtight container for further analysis.

2.4. Experimental

2.4.1. Organoleptic evaluation

The organoleptic evaluation of amalaki formulations was conducted for odor, color, appearance, and taste characteristics.

2.4.2. Physical parameter evaluation

The physical properties of amalaki formulations were analyzed using ayurvedic pharmacopoeial methods. [17]

2.4.3. Qualitative Phytochemical evaluation

Qualitative phytochemical evaluations of amalaki formulations were undertaken by different phytochemical tests viz. the

alkaline reagent test, Benedict's test, Libermann-Burchard test, Salkowski test, etc. [46,47]

2.4.4. Quantitative Phytochemical Analysis

2.4.4.1. Total phenolic content

The total quantity of phenolic content in amalaki formulations was determined using the Folin Ciocalteu method. [48,49] The gallic acid standard calibration curve was used for linear regression analysis. A test solution of extracts of amalaki formulation (100 mg/mL) was produced by diluting it with distilled water (1:15). In a test solution, 500 µL of Folin-Ciocalteu reagent (2 N) was added. After waiting for five minutes, 2 mL of washing soda (20%) was added to the volumetric flask. After one hour of incubation, the absorption was recorded in UV-VIS spectrophotometer at 765 nm.

2.4.4.2. Total flavonoid content

The aluminum chloride method determined the total quantity of flavonoids in amalaki formulations.[50] The quercetin standard calibration curve was used for linear regression analysis. First, 1 mL of stock solution (100 mg/mL) of amalaki formulation extract and 10% aluminum chloride (0.1 mL) were mixed in a volumetric flask. Then, 0.1 mL of 1M potassium acetate was incorporated into a mixture and kept for 1-2 minutes. Final 5ml volume made up of distilled water. After 30 minutes, the absorption was recorded in UV-VIS spectrophotometer at 430 nm.

2.4.4.3. Total Tannin Content

The Vanillin method estimated the total quantity of tannins in amalaki formulations.[51,52] The catechin standard calibration curve was used for linear regression analysis. 0.4 mL of stock solution (100 mg/mL) of amalaki formulation extract and 4 % vanillin solution (3 mL) were added to a volumetric flask. To a mixture, conc. Hydrochloric acid (1.5 mL) was mixed. After 15 minutes, the absorption was recorded in UV-VIS spectrophotometer at 500 nm.

2.4.4.4. Total Sugar content

The total quantity of sugar in amalaki formulations was estimated by phenol-sulfuric acid method. [53] The D-glucose standard calibration curve was used for linear regression analysis. In a volumetric flask, 1 mL stock solution (100 mg/mL) of extract of amalaki formulation, 1mL of 5% phenol, and vortexed after addition of 5 mL of conc. Sulfuric acid to the mixture. After 30 minutes, the absorption was recorded in UV-VIS spectrophotometer at 540 nm.

2.4.4.5. Total Reducing Sugar

The DNS reagent method estimated the total quantity of reducing sugar in amalaki formulations.[54] The D-glucose standard calibration curve was used for linear regression analysis. A test solution of extracts of amalaki formulation (100 mg/mL) was produced by diluting it with distilled water (1:3). In the mixture, DNS reagent (3 mL) was mixed and boiled for five minutes in a water bath. The absorption was recorded in UV-VIS spectrophotometer at 540 nm.

2.4.5. UV-Vis Spectrophotometric study

UV-Vis absorption spectra of extracts of amalaki formulation were recorded using Jasco UV Vis spectrophotometer in the spectral range 200 nm to 400 nm.

2.4.6. FT-IR study

The FT-IR spectra of the extract of amalaki formulations were recorded in the region 4000–400 cm^{-1} by the DRS method using a Shimadzu spectrometer.

2.4.7. HPTLC Analysis

2.4.7.1. TLC condition

The stationary phase used in TLC analysis was silica gel $60F_{254}$ pre-coated TLC plate (20 ×10 cm and 10 × 10 cm). For TLC plate development, the mobile phase consisted of toluene: ethyl acetate: methanol: formic acid (3: 3: 2: 1, v/v/v/v). The TLC glass chamber (20 X 10 cm) was used to saturate the mobile phase for 25 minutes. Then, the sample was applied on a TLC plate by a sample applicator with a microliter syringe ILS (100 µl) under a controlled nitrogen flow. The densitometric scanning of the TLC plate was done by WINCATS software using a CAMAG TLC Scanner.

2.4.7.2. Preparation of stock solution

The stock solutions of rutin, ascorbic acid, gallic acid, & kaempferol (1mg/ml) were prepared using methanol (AR grade).

2.4.7.3. Preparation of test sample

The extracts of the amalaki formulation (1 mg/ mL) were dissolved in methanol (AR grade).

2.4.7.4. Procedures

TLC plates were prewashed with methanol & before use, activated at 110 °C in a dry heat oven for 10 min. The extracts of amalaki formulations & markers were applied on previously prewashed, activated TLC plates by a sample applicator. The TLC plates were developed in a mobile phase containing toluene: ethyl acetate: methanol: formic acid (3: 3: 2: 1, v/v/v/v). The development distance of the TLC plate was kept up to 7 cm. The bands on the TLC plates were scanned under wavelength at 265 nm. The R_f values of each band were recorded.

3. METHODS VALIDATION

To ensure efficient chromatographic separation, an analytical method should be validated. The optimized HPTLC method was validated as per ICH guidelines Q2 (R1).[55]

3.1. Specificity

The markers & amalaki formulation were applied to the TLC plate to determine the specificity of the method. In addition, each chromatogram's peak start, apex, and end positions were examined to assess the peak purity of rutin, ascorbic acid, gallic acid, & kaempferol.

3.2. Linearity

On a TLC plate, working solutions of rutin (100–600 ng/band), ascorbic acid (200–1200 ng/band), gallic acid (100–600 ng/band), and kaempferol (100–600 ng/band) were separately applied. Then, the peak area v/s concentration of the applied markers was plotted to obtain a straight line, slope, and correlation coefficient (R2) equation.

3.3. Limit of detection and limit of quantification

The slope of the corresponding calibration curve and standard deviation (SD) of the peak areas of each marker were used to calculate the limits of detection and quantification.

3.4. Precision

Specify intraday precision was assessed by applying six replicates of the markers' three concentrations of working solutions to an HPTLC plate. The specific intraday precision was assessed for three different concentrations of each marker by applying six replicates of each concentration for different days.

3.5. Robustness

The small but deliberate variations in the volume of the mobile phase (± 2 mL) and duration of saturation time (± 2 min.) of the optimized HPTLC method were done to determine the robustness, and the finding was expressed in % RSD.

3.6. Accuracy

The recovery study of the rutin, ascorbic acid, gallic acid, & kaempferol standard addition technique was used to evaluate the method's accuracy. Rutin, ascorbic acid, gallic acid, & kaempferol were each added in three different amounts (400 ng/ band, 500 ng/ band, and 600 ng/ band) to the extract of amalaki formulation. The mixtures were analyzed for peak areas, and the percentage recovery was calculated.

4. QUANTIFICATION

Extracts of both amalaki formulations were applied in triplicate on previously prewashed TLC plates by a sample applicator. TLC plates were developed in an optimized mobile phase per section 2.5.7.4. The peak areas were noted for each marker. The content of each marker was calculated by linear regression obtained from the calibration curves.

5. STATISTICAL ANALYSIS

All statistical data were calculated using Microsoft Excel.

6. RESULTS & DISCUSSION

6.1. Organoleptic evaluation

The marketed amalaki formulation I & II were evaluated for organoleptic characteristics. Both amalaki formulations were brown solid & having a sour and astringent taste. As per ayurvedic pharmacopeia, amalaki formulations were identified & confirmed by the organoleptic evaluation, and results of the same are shown in Table 1.

Table 1: Results of Organoleptic Evaluation

Sr. No.	Particular	AMK I	AMK II
1	Appearance	Solid	Solid
2	Colour	Brown	Brown
3	Odor	Sour	Sour
4	Taste	Sour and astringent	Sour and astringent

6.2. Physical evaluation

The marketed Amalaki formulations I & II were evaluated for physical parameters. Both amalaki formulations were shown the values of foreign matter, moisture content, total ash, insoluble acid ash, alcohol soluble extractive value, and water-soluble extractive value are within the pharmacopoeial limit. In both amalaki formulations, no foreign matter is found, indicating its purity. Moisture content below 1 % confirmed that both amalaki formulations might store longer. The total ash value of both amalaki formulations was within the limit, which indicated that inorganic residue is less & it confirmed the quality of the formulations.[56–58] Both amalaki formulations had acid-insoluble ash values below 2%, indicating that a limited quantity of the inorganic component is acid-soluble, which confirmed that the amalaki formulations are not impure.[56–58] Phytoconstituents like sugars and mucilage, etc., are evaluated using a water-soluble extractive value & tannins, resins, and alkaloids are evaluated using an alcohol-soluble extractive value, and results of the same are shown in Table 2.

Table 2: Results of Physical evaluation

Sr. No.	Parameters	Specification as per Ayurvedic PI	AMK I	AMK II
1	Foreign matter	NMT 3 %	Nil	Nil
2	Moisture content	Not mentioned	0.70%	0.30%
3	Total ash	NMT 7 %	4.35 %	4.25%
4	Acid insoluble ash	NMT 2 %	1.80%	1.90%
5	Alcohol soluble extractive value	NTL 40 %	43.10 %	45.50%
6	Water soluble extractive value	NTL 50 %	40.10 %	41.50%

**** NMT:Not more than, NLT:- Not less than***

6.3. Qualitative Phytochemical Evaluation

The marketed amalaki formulations I & II were evaluated for qualitative phytochemical evaluation, and results of the same are shown in Table 3. These qualitative tests rely on color or precipitation reactions to indicate the presence of distinct chemical compounds.[59] It revealed that the extracts of amalaki formulations contained alkaloids, flavonoids, tannins, and carbohydrates.

Table 3: Results of Qualitative Phytochemical Evaluation

Sr. No.	Phytochemical	Test	Observation	AMK I	AMK II
1.	Alkaloids	Picric acid test	Yellow color	Positive	Positive
		Dragendorff's test	orange, red precipitate	Positive	Positive
2.	flavonoids	Alkaline reagent test	Intense yellow colour	Positive	Positive
		Shinoda test	deep pink colour	Positive	Positive
		Mayer's test	a yellowish or white precipitate	Positive	Positive
3.	Phenols	$FeCl_3$ test	Yellowish orange color	Positive	Positive
4.	Tannin	Lead sub-acetate test	gelatinous precipitate	Positive	Positive
5.	Carbohydrate	Benedict's test	Orange color	Positive	Positive
		Fehling's test	brick-red precipitate	Positive	Positive
		Molisch's test	purple colour	Positive	Positive
6.	Steroids	Libermann-Burchard test	No bluish-green color	Negative	Negative
7.	Terpenoids	Salkowski test	No intense red-brown color	Negative	Negative
8.	Coumarins	Sodium hydroxide solution Test	No dark yellow colour	Negative	Negative
9.	Saponins	froth formation Test	No froth formation	Negative	Negative

6.4. Phytochemical evaluation

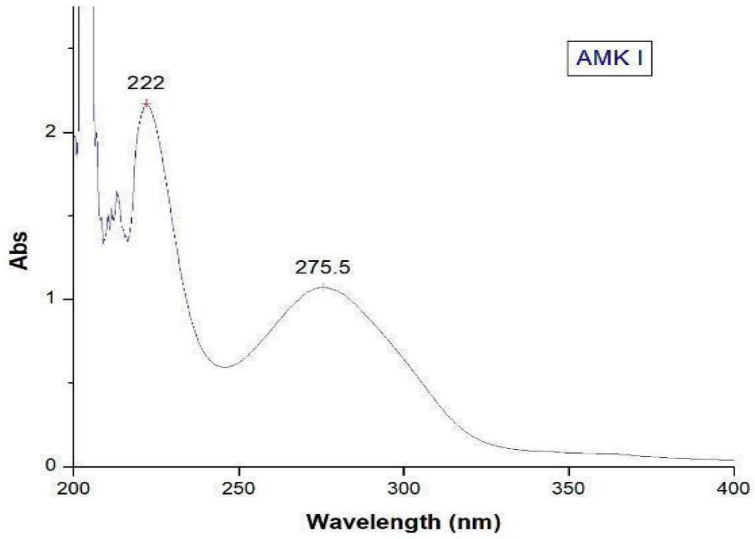
Phytochemicals viz. phenolic & tannins, and flavonoids are natural antioxidants that have the potential to be therapeutic agents for a variety of disorders such as neurological diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases, antibacterial, antiviral, antimutagenic activity and aging.[60-63] The extracts of amalaki formulations I & II were evaluated for phytochemicals by UV-Vis spectrophotometer using the standard curve & a linear regression analysis and reported in Table 4. It found that amalaki formulations are a rich source of phenolic compounds. Flavonoids were measured based on the development of complexes between flavonoids and aluminum.[50,64]Vanillin method was applied to determine the tannins based on the vanillin reaction with the phenolic rings of condensed tannins.[65,66] Total sugar determined by formation phenol furfural derivatives.[67] The reducing sugars are estimated by reducing the 3,5-dinitro salicylic acid reagent & sugar oxidized to sugar acid in an alkaline solution.[68]

Table 4: Results of Phytochemical Evaluation

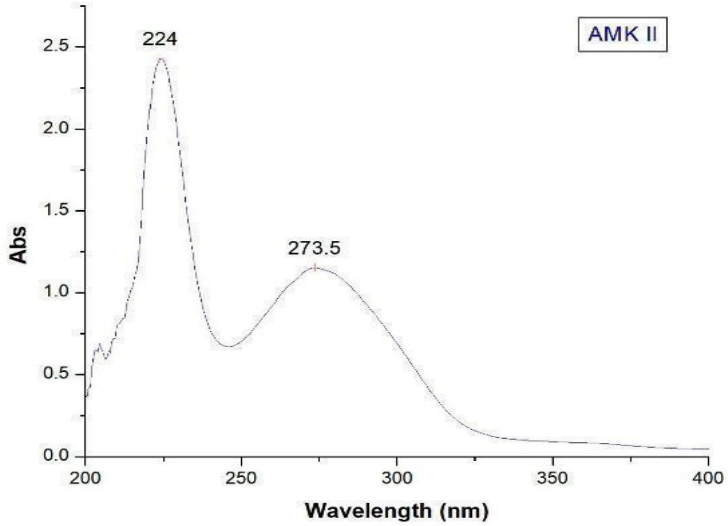
Sr. No.	Parameters	AMK I	AMK II
1	Total phenolic content	114.11±0.035mg GAE/g	116.12 ± 0.020 mg GAE/g
2	Total flavonoid content	29.15 ± 0.0029 mg QE/g	29.75 ± 0.0020 mg QE/g
3	Total Tannin content	4.50 ± 0.0085 mg CE/g	4.70 ± 0.0079 mg CE/g
4	Total Sugar content	7.34 ± 0.0034 mg Glu/g	7.44 ± 0.0043 mg Glu/g
5	Total Reducing Sugar Content	3.41 ± 0.0035 mg Glu/g	3.65 ± 0.0031 mg Glu/g

6.5. UV Vis Spectrophotometric study

The extracts of amalaki formulations I & II (10 µg/mL) were exposed to UV radiation between 200-400 nm. Extracts of amalaki formulations I and II showed two absorbance peaks in the UV-Vis spectra (Fig.1). Amalaki formulation I (AMK I) showed peaks at 222 nm & 275.5nm, and Amalaki formulation II (AMK II) showed a peak at 224 nm & 273.5 nm. The UV-Vis spectra of each amalaki formulation showed two band absorption spectra due to aromatic rings, which confirmed the presence of flavonoids and phenolic compounds.[65]



a) UV-Vis spectrum of AMK I

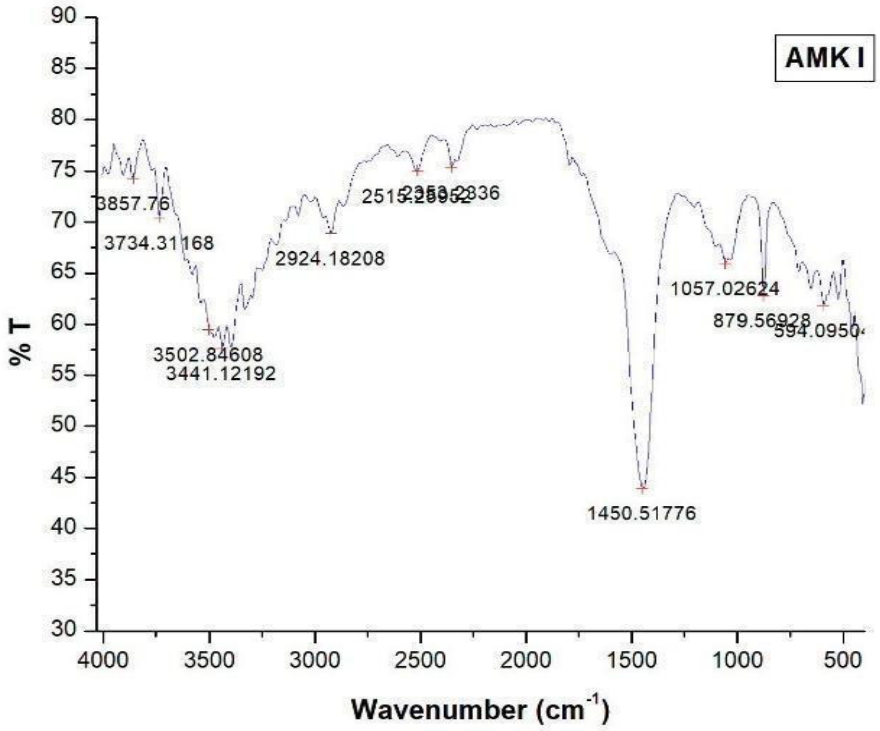


b) UV-Vis spectrum of AMK II

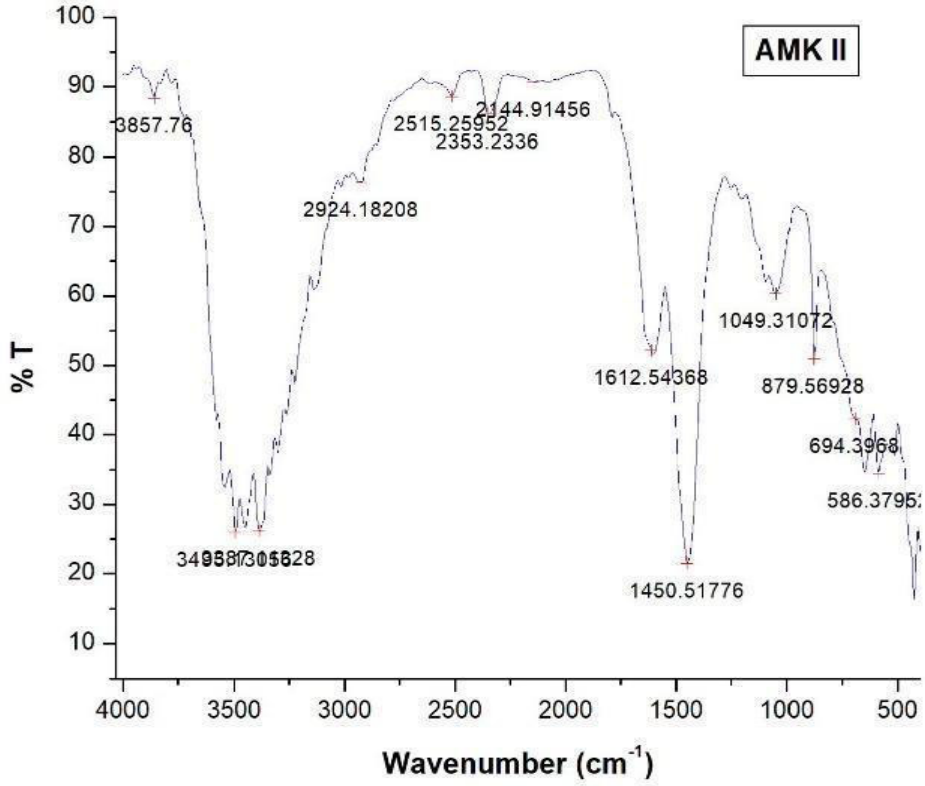
Fig.1. UV-Vis spectrum

6.6. FT-IR study

The solid-state IR (KBr, cm^{-1}) spectra of the amalaki formulation I& II extracts revealed a broad O-H group peak between 3520 to 3440 cm^{-1} and aromatic (C-H) stretch at 3100-3050 cm^{-1}. The stretching at 1615 to 1560 cm^{-1} confirmed the aromatic C=C group. The medium appearance of the vibration at 1455-1445cm^{-1} confirmed C-H bending. The stretching at 1058-1048 cm^{-1} revealed the presence of the C-O group. The peak between 865-880 cm^{-1} confirmed that C=C is bending.[68-70] (Fig 2)



a) FTIR spectrum of extract of AMK I



b) FTIR spectrum of extract of AMK II

Fig.2. FTIR spectrum of extract

6.7. HPTLC analysis

HPTLC analysis of amalaki formulations I and II provides accurate and precise Rf values and quantitative analysis by densitometry scanning. The chromatogram's densitometric scanning represents Rf values, peak area, and intensity. Each separated peak in the chromatogram indicated a phytochemical in the extract.[71,72]The extract from the amalaki formulations I and II was applied on precoated silica gel TLC plates. To develop HPTLC chromatograms of amalaki formulation, different combinations of different solvents were tried, and the combination of toluene, ethyl acetate, methanol, & formic acid (3: 3: 2: 1, v/v/v/v) was optimized as a mobile phase to separate the phytochemicals. The developed TLC plates were observed under 254 nm & 365 nm (Fig 3a & 3b). According to the chromatograms, there were eight to nine distinct phytochemicals in the extract of amalaki formulations. Nine bands with Rf values of 0.14, 0.24, 0.34, 0.50, 0.56, 0.68,

0.80, 0.87 & 0.90 were clearly shown by the HPTLC profile of amalaki formulation I (Fig. 3c) and eight bands with Rf values of 0.12, 0.17, 0.34, 0.50, 0.59, 0.80, 0.86 and 0.90 were clearly shown by the HPTLC profile of amalaki formulation II (Fig. 3d).Based on previous studies, we attempted to use the above optimized mobile phase, toluene, ethyl acetate, methanol, and formic acid (3: 3: 2: 1, v/v/v/v) for simultaneous estimation of various biomarkers such as Gallic acid, [70]kaempferol, rutin, quercetin,[71] ascorbic acid,[41,72] for extract of amalaki formulations. Rutin, ascorbic acid, gallic acid, and kaempferol were well-defined by densitometric scanning (Fig 3c & 3d). The rutin, ascorbic acid, gallic acid, and kaempferol showed well-defined bands at Rf values 0.34±0.015, 0.50±0.011, 0.80±0.015 & 0.90±0.015 under a wavelength of 265 nm.

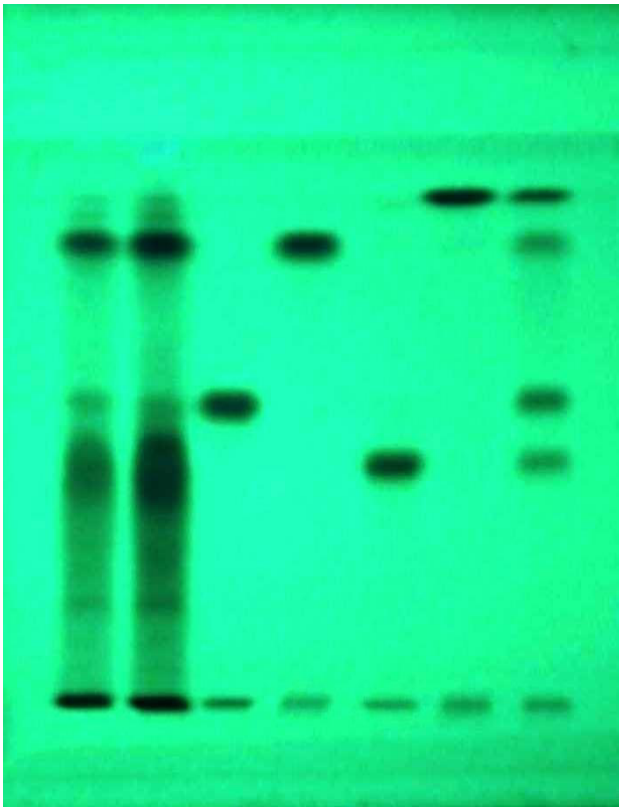


Fig 3: Photo documented plate a) 254nm

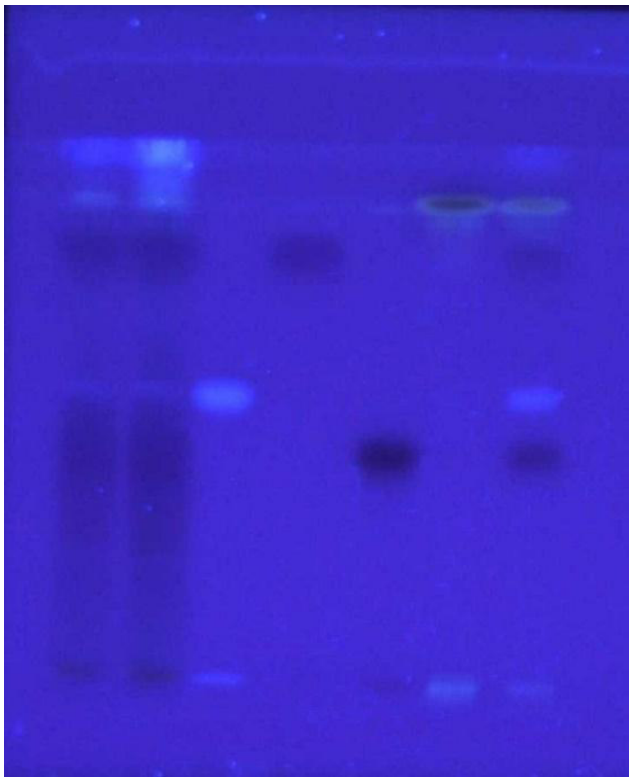


Fig. 3. Photo documented plate b) 365 nm

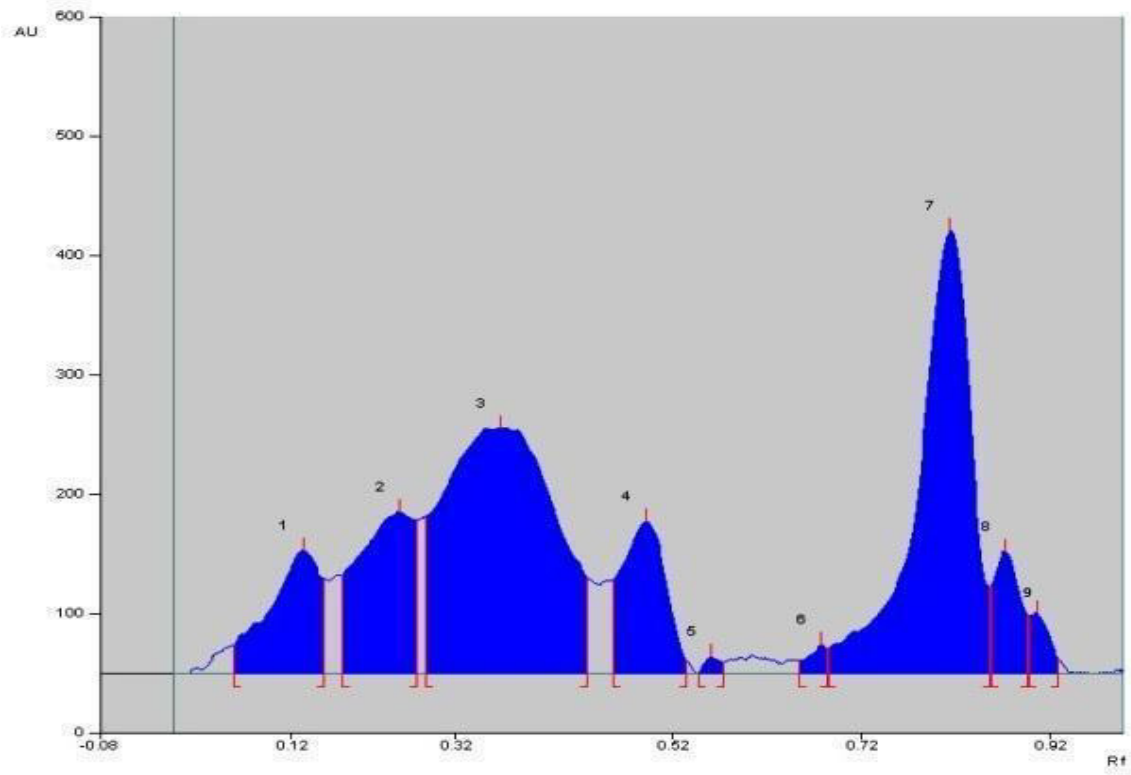


Fig. 3. c) Chromatogram of AMK I

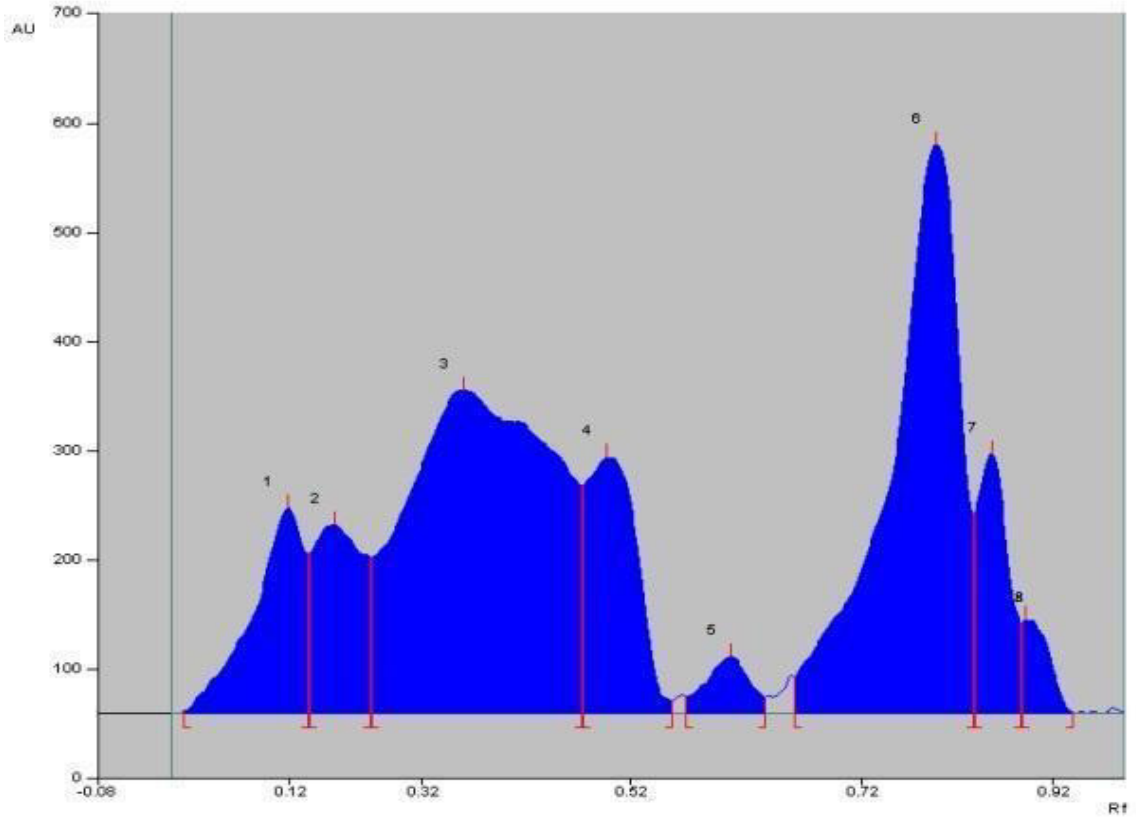


Fig. 3. d) Chromatogram of AMK II

6.8. Method validation

6.8.1. Specificity

In specificity, the extracts of amalaki formulations were compared with markers. The bands for rutin, ascorbic acid, gallic acid, and kaempferol in the extracts of amalaki formulations were confirmed by comparing the R_f and overlaying peak purity spectra with the markers.

6.8.2. Linearity

In linearity, the linear relationship between the different concentrations of the marker and their peak area response

was estimated. For rutin, gallic acid, and kaempferol, a linear correlation was achieved at concentrations of 100–600 ng/band; for ascorbic acid, it was 200–1200 ng/band. 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 5.

Table 5:- Linear Regression, LOD & LOQ

Parameters/ Markers	Rf	Linearity Range	Equation	R^2	LOD	LOQ
Rutin	0.3366±0.0152	100-600 ng/band	y = 3.4774x + 221.23	0.9996	30.21	91.56
Ascorbic Acid	0.4933±0.0115	200-1200 ng/band	y = 2.7789x - 122.48	0.9997	54.81	166.1
Gallic Acid	0.8033±0.0152	100-600 ng/band	y = 3.1858x + 22.403	0.9999	18.6	56.37
Kaempferol	0.9033±0.0152	100-600 ng/band	y = 3.076x + 95.44	0.9996	32.25	97.74

6.8.3. Limits of Detection and Quantification

For each marker, the limits of detection & quantification were calculated using the standard deviation method. The table displays the rutin, ascorbic acid, gallic acid, & kaempferol limits of detection & quantitation.

6.8.4. Precision

Intra-day precision & inter-day precision for the peak area of all markers was performed by repeated analysis. The % RSD of each marker was less than 2, demonstrating the method's precision. And shown in Table No.6.

Table 6:- Precision

Parameters/ Markers	Intra-day				Inter-day		
	Concentration	Mean Area (n=6)	SD	%RSD	Mean Area (n=6)	SD	%RSD
Rutin	100 ng/band	579.73	6.021	1.038	582.05	5.951	1.022
	300 ng/band	1261.67	12.827	1.016	1261.78	13.89	1.1
	500 ng/band	1952.84	17.724	0.9076	1954.2	18.412	0.9422
Ascorbic Acid	200 ng/band	431.64	4.924	1.14	432.125	5.083	1.176
	600 ng/band	1550.4	15.79	1.018	1548.47	16.173	1.044
	1000 ng/band	2656.4	19.59	0.737	2650.62	20.6	0.777
Gallic Acid	100 ng/band	339.95	4.569	1.344	343.13	5.039	1.468
	300 ng/band	961.08	8.71	0.906	961.47	10.66	1.109
	500 ng/band	1629.03	11.16	0.685	1628.25	11.38	0.699
Kaempferol	100 ng/band	387.4	3.97	1.026	385.27	4.05	1.052
	300 ng/band	1018.56	8.035	0.788	1019.48	8.394	0.823
	500 ng/band	1625.32	9.32	0.82	1628.85	12.91	0.79

6.8.5. Robustness

The method was found to be robust regarding changes in the volume of the mobile phase (± 2 mL) and duration of saturation time (± 2 min.). The results obtained for robustness studies and % RSD are shown in Table 7.

Table 7: Robustness

Parameters/ Markers	Concentration	Mobile Phase Volume (±2 mL)	Mean Area	SD	%RSD	Duration of Saturation Time (±2 min)	Mean Area	SD	%RSD
Rutin	200 ng/ band	8 mL	895.1	11.22	1.215	23 min	896.6	11.21	1.236
	200 ng/ band	10 mL	900.27	9.27	1.031	25 min	902.3	8.24	1.012
	200 ng/ band	12 mL	897.5	10.24	1.246	27 min	899.5	10.27	1.428
Ascorbic Acid	200 ng/ band	8 mL	425.1	6.24	1.261	23 min	427.32	7.67	1.211
	200 ng/ band	10 mL	430.7	4.9	1.140	25 min	431.1	7.11	1.196
	200 ng/ band	12 mL	429.25	7.51	1.289	27 min	428.7	7.89	1.576
Gallic Acid	200 ng/ band	8 mL	641.6	9.21	1.413	23 min	650.23	10.79	1.359
	200 ng/ band	10 mL	659.11	8.71	1.017	25 min	657.76	8.24	1.011
	200 ng/ band	12 mL	647.7	10.25	1.275	27 min	654.27	9.43	1.262
Kaempferol	200 ng/ band	8 mL	707.3	9.25	1.227	23 min	710.3	8.32	1.225
	200 ng/ band	10 mL	713.5	8.37	1.176	25 min	715.41	7.19	1.129
	200 ng/ band	12 mL	719.7	9.72	1.266	27 min	713.2	7.51	1.341

6.8.6. Accuracy

The extract of amalaki formulations was spiked with the known amount of markers, and the percent recovery was calculated. The results obtained are shown in Table. 8.

Table 8: Recovery studies

		% Recovery ± SD	
Parameters/ Markers	**Amount of Standard Added (ng/ band)**	**AMK I**	**AMK II**
Rutin	400	99.95±0.76	99.94±0.75
	500	99.97±0.31	99.93±0.47
	600	99.98±0.65	99.76±0.71
Ascorbic Acid	400	99.91±0.47	99.87±0.41
	500	99.98±0.73	99.67±0.66
	600	99.62±0.45	99.57±0.79
Gallic Acid	400	100.01±0.73	100.09±0.71
	500	99.99±0.79	100.07±0.57
	600	99.99±0.63	99.97±0.49
Kaempferol	400	99.09±0.63	99.92±0.73
	500	99.79±0.69	99.87±0.42
	600	99.29±0.71	99.89±0.81

7. QUANTIFICATION

The developed HPTLC method was used to estimate rutin, ascorbic acid, gallic acid, and kaempferol in the extract of amalaki formulations. The densitometry scanning was performed for each marketed amalaki formulation. The amount of rutin, ascorbic acid, gallic acid, and kaempferol was calculated by linear regression, and the result is shown in Table 9.

Table 9: Quantification of markers

	Quantification	
Parameters/ Markers	**AMK I (g/100 gm)**	**AMK II (g/100 gm)**
Rutin	0.861±0.097	1.174±0.107
Ascorbic Acid	0.4356±0.042	0.5392±0.047
Gallic Acid	0.812±0.098	1.05±0.102
Kaempferol	0.106±0.110	0.160±0.29

8. CONCLUSION

The present research used organoleptic, physical, phytochemical, spectroscopic, and chromatographic studies to evaluate the amalaki formulations (AMK I & II). UV-Vis spectrophotometric methods were utilized to determine the phytochemical contents of amalaki formulations by linear regression method. The phytochemical evaluation indicates that amalaki formulations contain significant amounts of flavonoids, tannins, and phenolic compounds. In addition, the FTIR spectra of amalaki formulations indicated the presence of several functional groups of complex phytochemicals. For the qualitative and quantitative examination of the rutin, ascorbic acid, gallic acid, and kaempferol in the commercial amalaki formulations, the developed HPTLC technique was shown to be Simple, accurate, and reliable. The normal analysis of the amalaki formulation can be done using this approach.

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10. AUTHOR'S CONTRIBUTION STATEMENT

Mr. Ganesh B Nigade conceptualized and discussed the methodology and results in the final manuscript. Dr. Meenakshi Deodhar and Dr. Rajashree Chavan supervised the research work.

11. CONFLICT OF INTEREST

Conflict of interest declared none.

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