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Original Article

Physicochemical and HPTLC Estimation of Gallic Acid and Ascorbic acid in *Amalaki Avaleha*

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ABSTRACT
Amalaki avaleha an ayurvedic formulation that highlights the *Rasayana* and blood enhancing properties of *Amalaki*. *Amalaki Avaleha* is mentioned in *Sushruta samhita* Uttar Tantra in reference of *Pandu roga* (Anemia). This study is based on the HPTLC analysis of two active Phytochemicals constituents' Gallic acid and Ascorbic acid present in *Amalaki avaleha* to authenticate its phytochemical profile and substantiate its therapeutic claims. Pre-coated silica gel 60 F_{254} plates used for evaluation. Gallic acid was examined with a solvent system incorporated by Ethyl acetate, Acetone, Water and Formic acid (10:6:2:2 v/v/v/v) resulting in a distinctive Rf value of 0.89 and a Coefficient of Variation (CV) of 2.81%, indicating precision and reproducibility. Ascorbic acid was evaluated using a solvent system of Ethanol, Glacial acetic acid and Toluene in a ratio of (3.5:1.5:3 v/v/v), which showed an Rf value of 0.603 and a CV of 5.03%. These results confirm that the Gallic acid and ascorbic acid is present in the *Amalaki Avaleha*, aligning with its traditional application in blood-related conditions. The confirmed HPTLC method offers a dependable and consistent means for the phytochemical standardization of this historically recognized formulation, guaranteeing its identity, quality, and therapeutic effectiveness.
Keywords: HPTLC, Gallic acid, Ascorbic acid, Amalaki Avaleha, Mobile phase, Solvent system.

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INTRODUCTION

Amalaki Avaleha mentioned in the 44th Chapter of *Pandu roga pratishedhan Adhyaya* of *Uttartantra* in *Sushruta Samhita*, which is mentioned in context of *Pandu roga* (Anemia) treatment. *Amalaki Avaleha* consists of *Amla* and *madhu*, mixed in a ratio of one to three *masha* (amount), using *amla churna* with *madhu*.[1] *malaki Avaleha* is set be rasayana in ayurved i.e. it helps in nourishes all 7 types of *dhatu* (essence of body tissue), *Vayasthapana* (Anti-agening), *Rasayana* (rejuvenator), Enhances *Oja* (Physical vitality & immunity), Immunity, Support cognitive eye health.[2] There are six *rasas* according to ayurveda but in *Amalaki Avaleha* contains five rasas (*amla, madhura, katu, tikta, kashaya*). These rasa balances *vata, pitta* and *kapha dosha*. *Amla* commonly called the Indian gooseberry (*Emblica officinalis* Gaertn) is a fruit that originates from India and has benefits for health as well as nutritional content. This fruit belongs to the *Euphorbiaceae* family and is often labeled a miracle fruit for wellness.[3]

The fruits of *Amla* are widely used in Ayurvedic formulations and are believed to improve the body's resistance to diseases. It is an exceptional dietary supplement that offers a variety of health benefits.[4] The Indian gooseberry is included in Ayurvedic preparations and aids in strengthening defenses against illnesses.[5] This substance contains a variety of chemical compounds including tannins, alkaloids and phenols.[6] Among all hydrolysable tannins, Emblicanin A and B, along with gallic acid and ellagic acid are noted for their biological efficacy. Almost every part of the plant possesses medicinal properties, particularly the fruit, which has been employed in Ayurveda as a powerful *rasayana* and is used in traditional medicine to address conditions such as diarrhea, jaundice, inflammation and several others.[7]

Honey is a naturally occurring sweet substance made by honeybees from the nectar of flowers as well as the excretions of plant-sucking insects on those parts, which the bees gather, transform by mixing with special substances they produce, deposit, dehydratestore and allow maturing and fermenting in honeycombs.[8] It is a natural substance resulting when honeybees from the Apis and Meliponini genera collect, modify and stash nectar and sugary plant materials in their honeycombs.[9] Honey has medicinal properties and its products obtained directly from the hive also provided health benefits, a practice referred to as "apitherapy".[10] *Madhu* functions synergistically as *Yogavahi*, making it the optimal *Anupana - Dravya* to be consumed with or after taking *Aushadha* (medication). It follows the *Sukshamamargaanusari* principle, indicating its capacity to infiltrate the *Srotas* (microchannels). It is extensively utilized in *Navjaat Shishu Parichrya* (newborn care rituals).

Gallic Acid (GA), which is categorized as a type of phenolic compound, is also referred to as 3,4,5-trihydroxybenzoic acid (Figure 1). It appears as a colourless to slightly yellow crystalline substance and utilized in both the food and pharmaceutical industries.[11] Among various polyphenols, Gallic acid is a small molecular weight tri-phenolic compound known for its remarkable anti-inflammatory and antioxidant properties,[12] as well as its anticancer effects.[13] Pharmacological studies concerning gallic acid highlight its antimicrobial properties,[14] cardiovascular benefits,[15] gastrointestinal effects,[16] metabolic activities,[17] neuropsychological

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impact,[18] protection against radiation-induced toxicity,[19] respiratory benefits,[20] urinary functions,[21] urogenital health,[22] dermal applications[23] and its role in malignancy enzymes.[24]

L-Ascorbic acid (L-threo-hex-2-enono-1,4-lactone, ascorbate) (Figure 2), commonly referred to as Vitamin C, is an essential antioxidant in both plant and animal systems and functions as a co-factor for various enzymes.[25] The hydroxylation process performed by these enzymes requires an Fe^{2+} ion at the active site, which gets oxidized to Fe^{3+} during the catalytic cycle, while ascorbate is essential for restoring the iron's activity by converting it back to Fe^{2+}. Fresh fruits and vegetables are the main sources of this vitamin, so increasing its levels can significantly affect human nutrition.[26] The use of intravenous Ascorbic Acid (AA) as an adjunct therapy in cancer treatment has been investigated since the 1970's.[27-29] High-dose AA has been studied as a potential cancer treatment since the 1970's.[27] Results from more recent clinical studies demonstrate that intravenous Vitamin C is safe for cancer patients, with very few adverse effects noted.[30] Further research indicates that combining high-dose Vitamin C with cancer treatments may slow tumour development in models of pancreatic,[31,32] liver,[33] prostate,[34] ovarian[35] and sarcoma cancers.[36]

The main goal of this research was to quantify the levels of Gallic acid and Ascorbic acid in *Amalaki Avaleha* by using the HPTLC technique and to develop a quality control method for assessing its effectiveness. Additionally, a physicochemical analysis was conducted to evaluate the physiochemical properties (Organoleptic character, Loss on drying (LOD), Total ash, Acid insoluble ash, Alcohol soluble extractive, Water soluble extractive, pH) of a substance or system in order to comprehend its composition, behaviour and potential effects.

MATERIALS AND METHODS

Plant Material and Formulations

The fruit of *Emblica officinale* Geartn. was collected from the local drug supplier in Raipur, Chhattisgarh. The sample was authenticated by Shri B.M.K. Ayurveda Mahavidyalaya, which is part of KLE Academy of Higher Education and Research Central Research Facility in Belgaum, Karnataka on 27 March 2024 (Ref: BMK/CRF/87/2024-25). Amalaki *Avaleha* (Table 1) was prepared at the Government Ayurveda Pharmacy in Raipur, under the supervision of the *Ras Shastra AvamBhaisajya Kalpana* department in June 2024. The preparation method of *Amalaki avaleha* is mentioned in *Sushrut Samhita* in context of *panduroga* (Figure 3).

Physicochemical Analysis of *Amalaki Avaleha*

The raw material of *Amalaki Avaleha* and final formulation of *Amalaki Avaleha* are underwent testing at the Drugs Testing Laboratory Avam Anusandhan Kendra in Raipur, Chhattisgarh, following the protocols outlined in the Ayurvedic Pharmacopoeia of India (Tables 2,3 and 4).[48-50]

Chemical and reagents

Gallic acid, Ascorbic acid and precoated TLC Silica-G aluminium 60 F254 plate were obtained from the Drugs Testing Laboratory Avam Anusandhan Kendra located in Raipur (CG), India. Methanol was sourced from Loba Chemical Pvt. Ltd. for HPTLC purposes. All other chemicals, reagents and solvents were utilized in their Analytical grade.

Preparation of the test sample

For preparation of 50 mg/mL sample solution, 500 mg of sample was dissolved using 10 mL of methanol. Subsequently, the extract was sonicated for 10 minutes. Thereafter, filter the solution through a 0.2µm syringe-filter to produce a clear solution for HPTLC analysis.

Standard Gallic acid, Ascorbic acid solution

Standard Gallic acid and Ascorbic acid

To prepare standard solutions, 10 mg each of Gallic acid and Ascorbic acid were separately dissolved in 10 mL of methanol, resulting in a concentration of 1 mg/mL. The solutions were then sonicated for duration of 10 min. Finally, the stock solutions were filtered through 0.2 µm syringe filters to remove any suspended particles.

Preparation of Mobile phase

Mobile Phase

The solvent system employed for the separation and examination of the target analyze involves different compositions. For Gallic acid, a mixture consisting of Ethyl acetate, acetone, water, and formic acid in a volume ratio of (10:6:2:2, v/v/v/v) is used, while for Ascorbic acid, a combination of ethanol, glacial acetic acid, and toluene in the proportion of (3.5:1.5:3, v/v/v) is applied.

Instrumentation and Chromatographic parameters

GA and Ascorbic acid were identified and measured in *Amalaki Avaleha* using the CAMAG HPTLC system. The separation was carried out on a TLC plate coated with silica gel F60254 (200 × 100 mm) and having a thickness of 0.25 mm. All solutions were applied using the Linomat 5 automatic sample spotter with a 7 mm bandwidth, track distance between bands 11.4 mm and positioned away from the bottom edge. The application rate was consistently maintained at 150 nL/s. The plate was elevated to a height of 70 mm within a CAMAG twin-trough glass chamber. Before development, the glass chamber was saturated with the vapour of the solvent system for 20 minutes at 25 °C and then developed separately using a Gallic acid solvent mixture consisting of Ethyl acetate: Acetone: Water: formic acid (10:6:2:2 v/v/v/v) and an Ascorbic acid solvent mixture composed of Ethanol: Glacial acetic acid: Toluene (3.5:1.5:3 v/v/v). The visualization of the plate was carried out using Visualiser 2 (CAMAG, Switzerland). Densitometric scanning was performed using UV light, 254 and 366nm with TLC scanner 4 (CAMAG, Switzerland), utilizing a slit dimension of 5×0.2 mm and a scanning speed of 20 mm/s. The densitometric data were evaluated using the Vision CATS system.

Calibration curve of Gallic Acid (GA) and Ascorbic acid

Following earlier descriptions, volumes of GA and ascorbic acid at concentrations of 1.5µg, 2.0µg, 3.0µg, and 3.5µg were deposited onto the silica gel 60 F254 plate. The plate was set up and analysed to create a calibration equation for measuring the amount of Gallic acid in the samples (Figures 4 and 5).

Method validation

The process for validating the analytical methods adhered to the guidelines set forth by ICH.[51] The method was determined to be accurate, precise and consistently reproducible. The methods repeatability was tested by repeatedly scanning the same samples of Gallic acid and Ascorbic acid, with the results shown as coefficients of variation of 2.81% and 5.02%, respectively. Samples of Gallic acid and Ascorbic acid (1.5-3.5 µg) were analysed multiple times to evaluate the variability of the method. The results were expressed as a per Tentage of the Confidence Interval (CV). To determine the accuracy of the method, the recovery percentage and the mean recovery percentage were calculated.

Table 1: **Ingredients of Amalaki Avaleha.**

Sl. No.	Drug Name	Latin Name	Parts use	Chemical Constituents	Uses
1.	*Amalaki*	*Emblica officinale* Geartn.	Dried Pulp	Gallic acid, Ascorbic acid[37]	Antioxidant,[38] Cardioprotective,[39] Anti - diabetic activity[40] Anticancer activity[41]
2.	*Madhu*	-	Animal origine (Bee product)	Dextrose, Levulose, Sucrose[42]	Anti-inflammatory[43] Antioxidant activity[44] Wound healing activity[45] Anti - diabetic properties[46] antimicrobial properties[47]

Table 2: **Physicochemical Analysis of *Amla*.**

Sl. No.	Drug	Water soluble extractive (%)		Alcohol soluble extractive (%)		LOD (%)		Acid insoluble ash (%)		Total Ash (%)	
		Result	API	Result	API	Result	API	Result	API	Result	API
1.	*Amla*	28.8 %	NMT50	34 %	NMT 40	12%	NMT 3	0.33%	NMT 2	1.6%	NMT 7

Table 3: **Physicochemical analysis of *Madhu*.**

Sl. No.	Drug	Aniline Chloride test		Weight per mL at 250 c		Fiehe's Test		Loss on drying (%)		Total Ash (%)	
		Result	API	Result	API	Result	API	Result	API	Result	API
1.	Madhu	Negative	Negative	1.36	NLT 1.35	Negative	Negative	23	NMT 25	0.33	NMT 0.50

(NMT- Not more than, NLT- Not less than).

Table 4: **Physicochemical Parameters of *Amalaki Avaleha***

Sl. No.	Parameter studied	Result
1.	Loss on Drying (LOD) (%)	12
2.	Total Ash (%)	1.6
3.	Acid -insoluble ash (%)	0.33
4.	Alcohol soluble extractive (%)	34
5.	Water soluble extractive (%)	28.8
6.	pH	3.32

Table 5: **Concentration of Gallic acid in *Amalaki Avaleha*.**

Sl. No.	Sample	Concentration of Gallic acid (µg/mL)
1.	Track 1	215.9 (µg/mL)
2.	Track 2	222.7 (µg/mL)
3.	Track 3	229.1 (µg/mL)
4.	Track 4	244.6 (µg/mL)

Table 6: **Concentration of Ascorbic acid in *Amalaki Avaleha*.**

Sl. No.	Sample	Concentration of Ascorbic acid (µg/mL)
1.	Track 5	84.25 (µg/mL)
2.	Track 6	85.88 (µg/mL)
3.	Track 7	89.61 (µg/mL)
4.	Track 8	93.75 (µg/mL)

RESULTS

The HPTLC analysis was conducted utilizing a developing solvent system for Gallic acid, which formed of a combination of Ethyl acetate, Acetone, Water and formic acid in a proportion of (10:6:2:2, v/v/v/v) later on for ascorbic acid, a solvent system composed of Ethanol, Glacial acetic acid and Toluene in a part of (3.5:1.5:3, v/v/v). The HPTLC technique effectively quantified gallic acid and ascorbic acid in *Amalaki Avaleha,* yielding an Rf value of 0.89% for gallic acid with a coefficient of variation of 2.81%, and an Rf value of 0.603% for ascorbic acid, presenting a coefficient of variation of 5.02%. In *Amalaki Avaleha,* the concentration of gallic acid phytoconstituents in tracks 1, 2, 3, and 4 were recorded as 215.9 (µg/mL), 222.7 (µg/mL), 229.1 (µg/mL), and 244.6 (µg/mL), respectively, while ascorbic acid phytoconstituents in tracks 5, 6, 7, and 8 were noted as 84.25 (µg/mL), 85.88 (µg/mL), 89.61 (µg/mL), and 93.75 (µg/mL), individually (Tables 5 and 6). The process achieved a linear range of 1.5-3.5 µg/band, demonstrating high accuracy and minimal variability (Figures 6-12).

DISCUSSION

Amalaki Avaleha is an Ayurvedic preparation consisting of Amla and madhu, derived primarily from *Emblica officinalis* Geartn. *Amalaki Avaleha* celebrated for its abundant ascorbic acid (vitamin C) and gallic acid content, which are both key to its rejuvenative (Rasayana) and

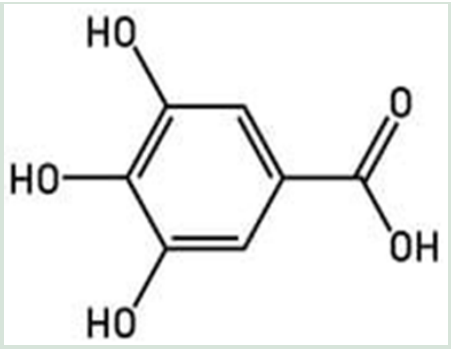


Figure 1: Chemical structure of Gallic acid (3,4,5-trihydroxy benzoic acid).

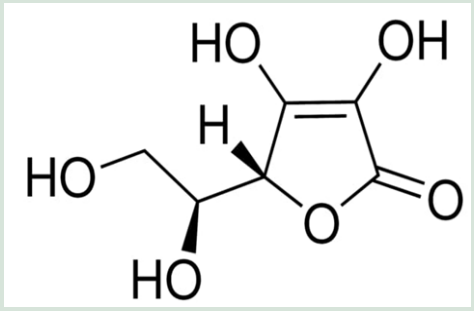


Figure 2: Chemical structure of Ascorbic acid ($C_6 H_8 O_6$).

सालादिकं चाप्यथ सारचूर्णं धात्रिफलं वा मधुना अवलिह्यात् ।

(सु उ.44/30) पाण्डुरोगप्रतिषेधं व्याख्यास्याम)

Figure 3: Preparation of *Amalaki Avaleha* according to *Sushrut Samhita* (S.S.).

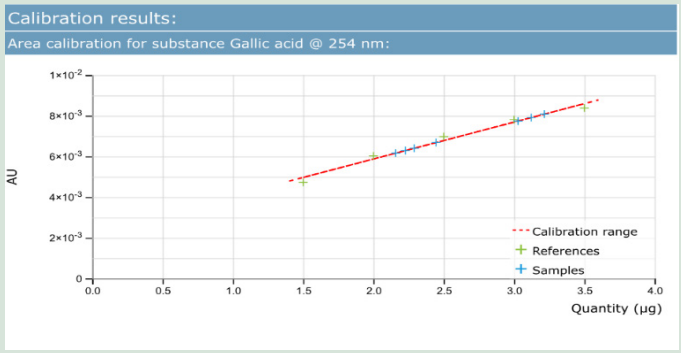


Figure 4: Calibration curve of Gallic acid.

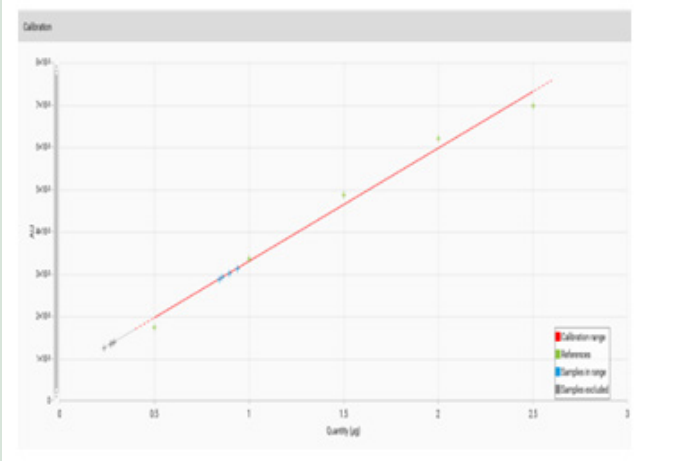


Figure 5: Calibration curve of Ascorbic acid.

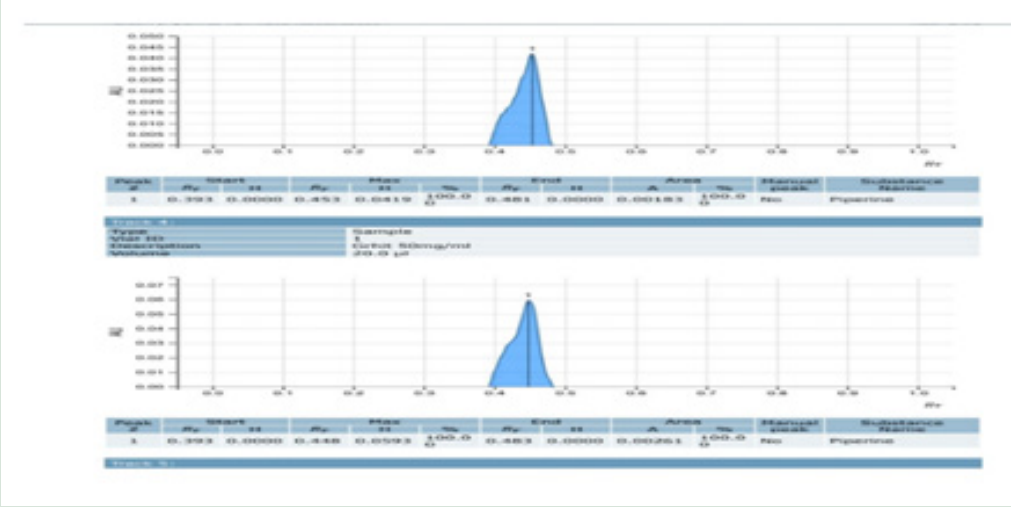


Figure 6: HPTLC Chromatogram of Gallic acid.

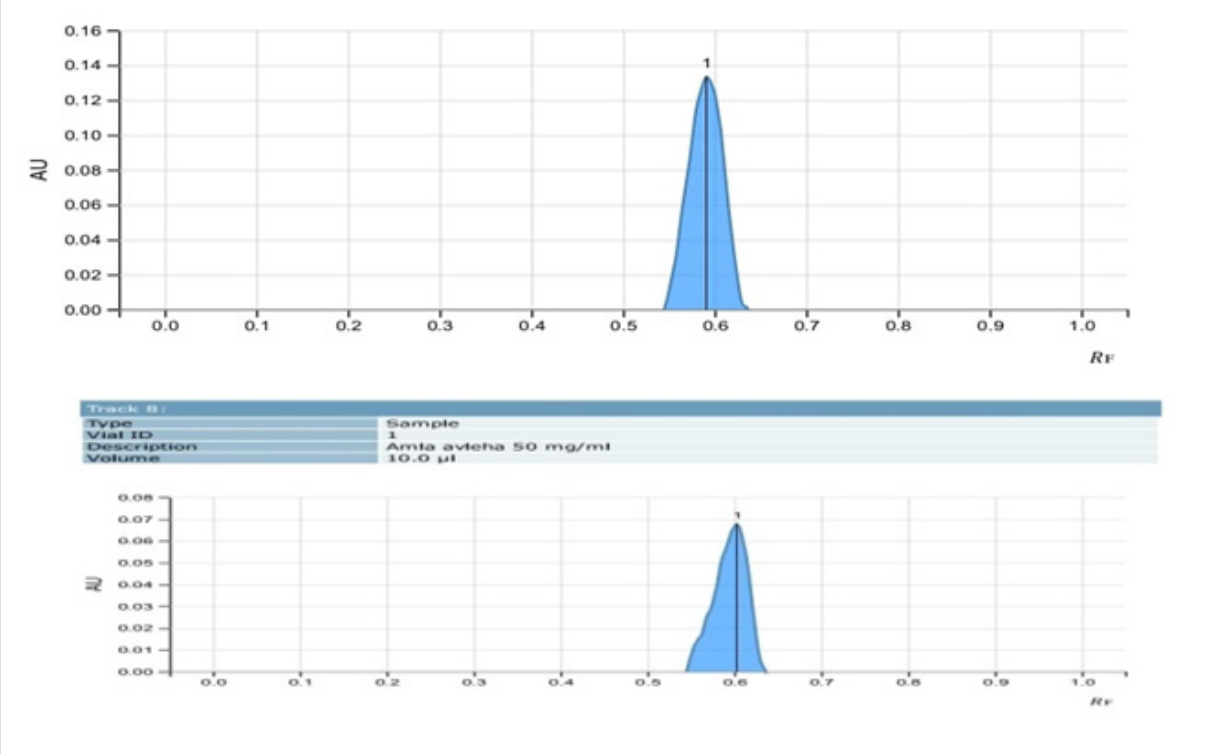


Figure 7: HPTLC Chromatogram of Ascorbic acid.

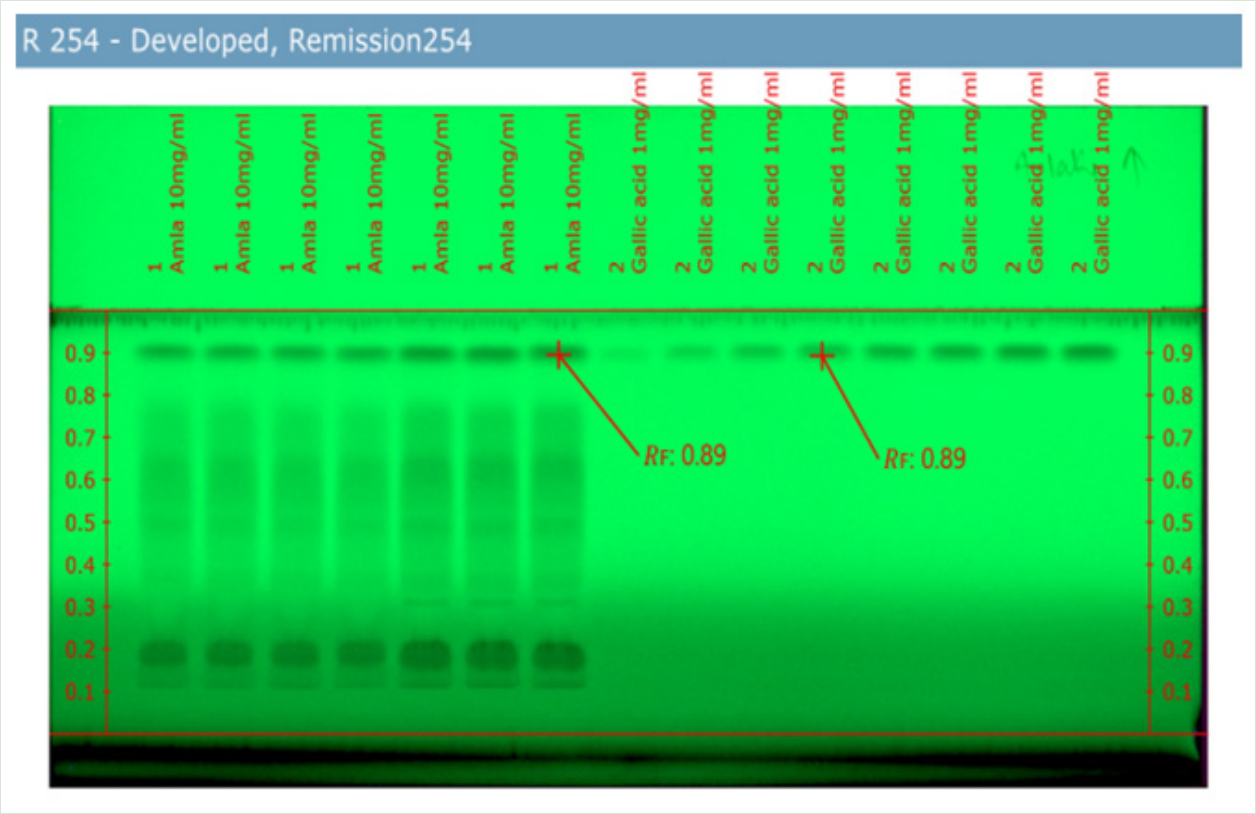


Figure 8: TLC Fingerprint of Gallic at 254 nm.

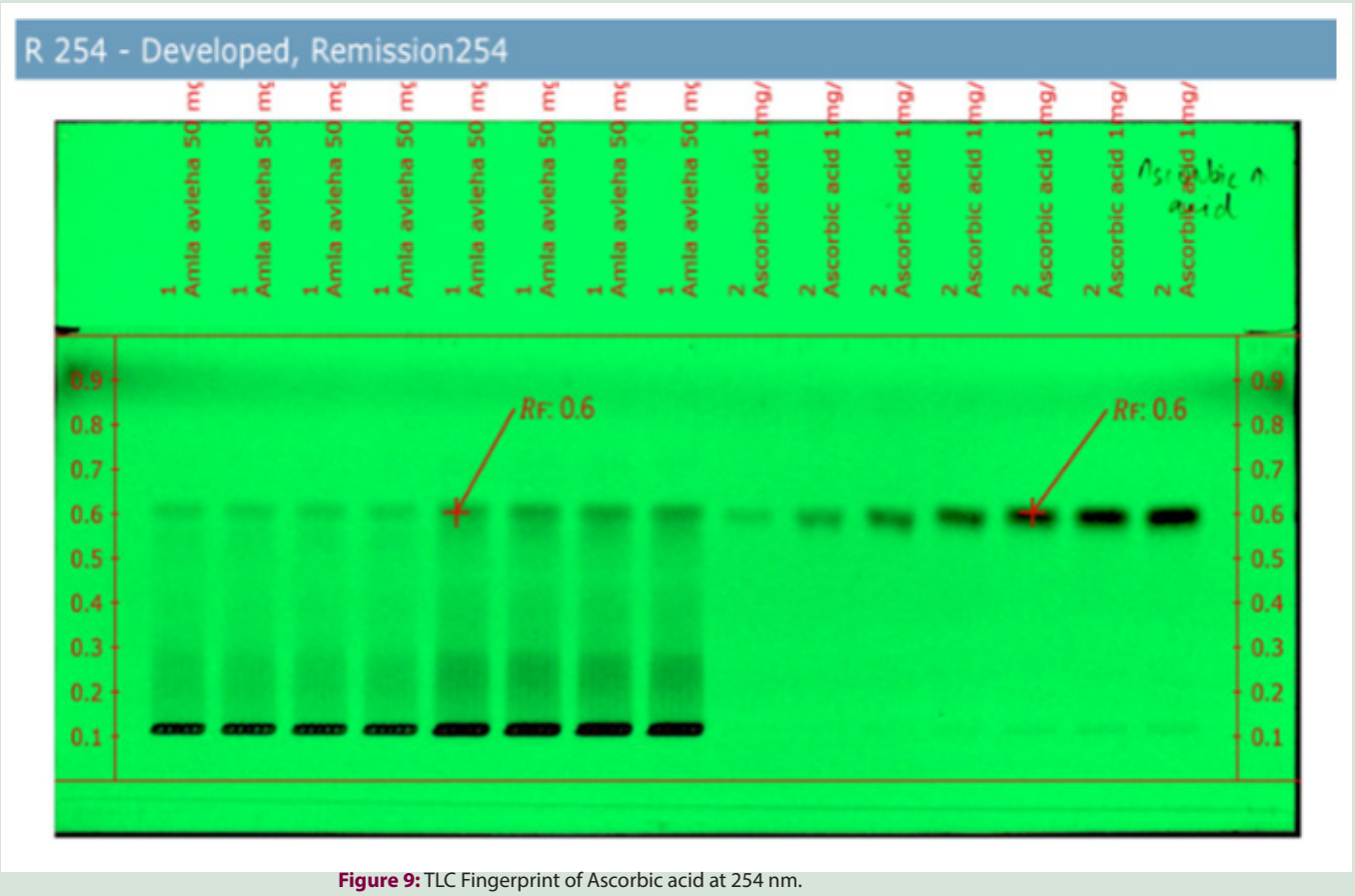


Figure 9: TLC Fingerprint of Ascorbic acid at 254 nm.

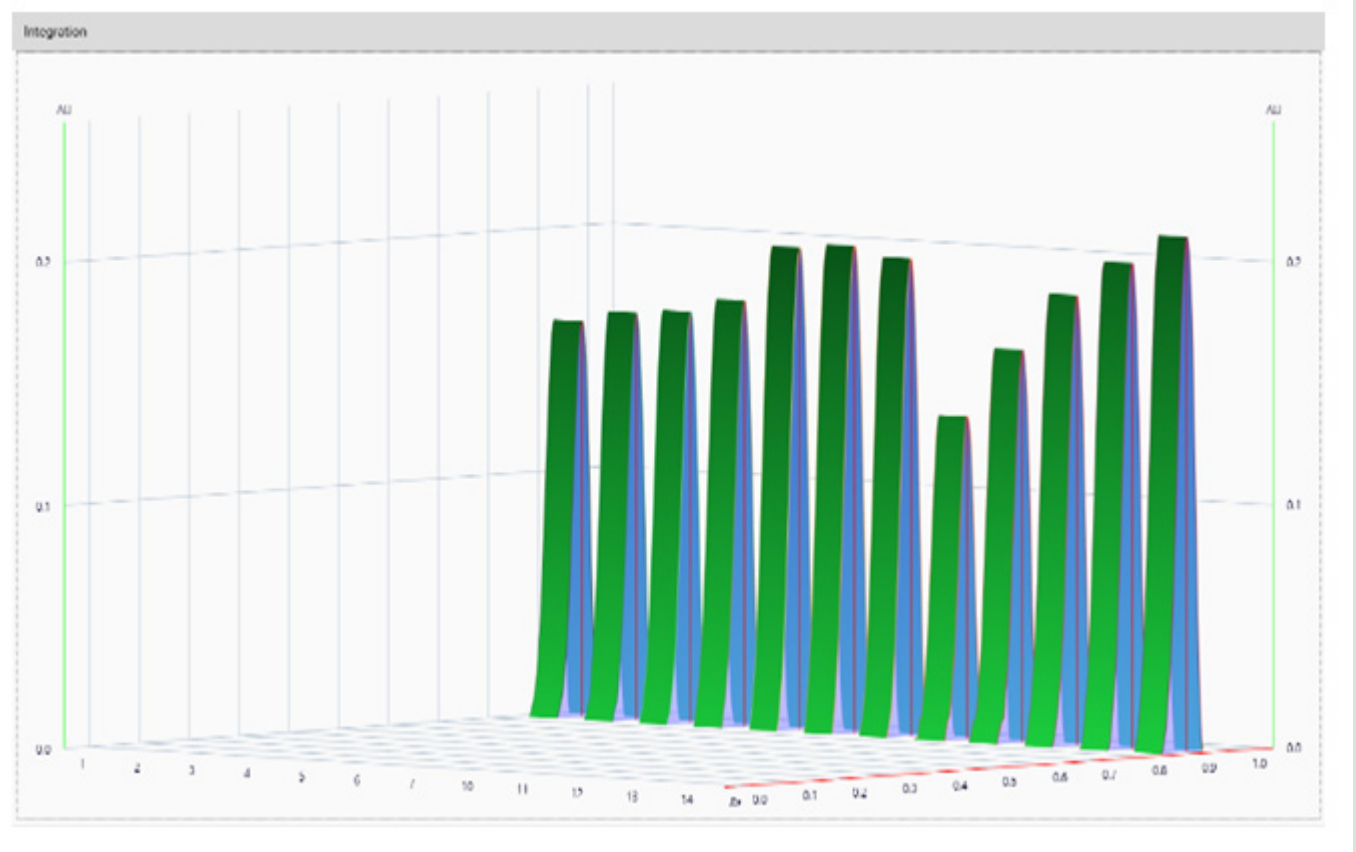


Figure 10: Fingerprint analysis of Gallic acid.

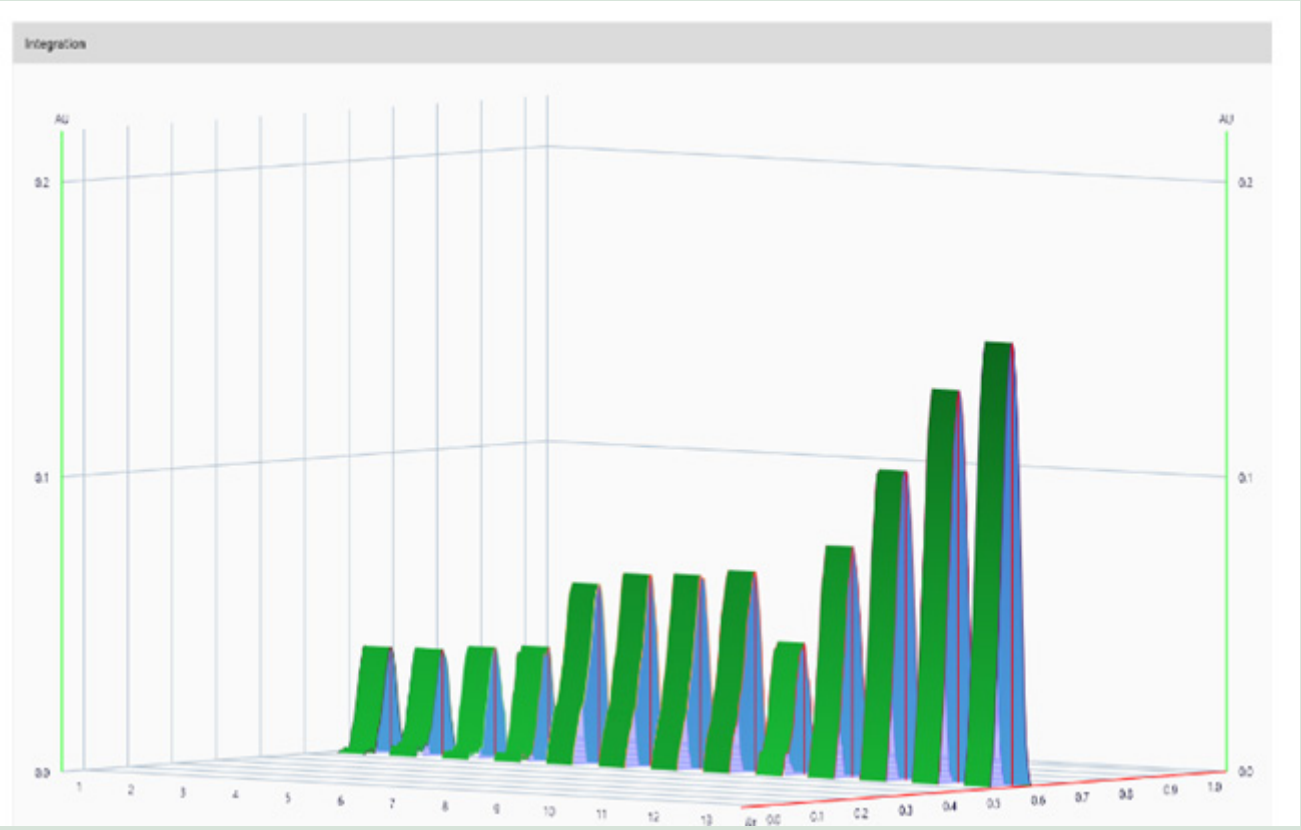


Figure 11: Finger print analysis of Ascorbic acid.

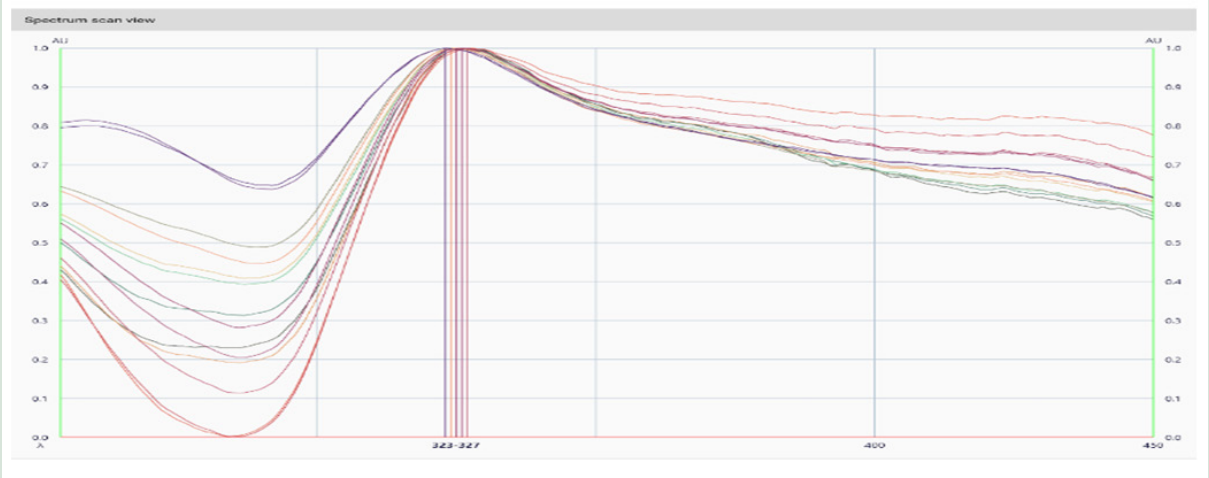


Figure 12: Spectrum analysis of Gallic acid.

antioxidant properties. This formulation leverages the rejuvenating and blood-enhancing qualities of *Amla* (*Emblica officinalis* Geartn*)*. HPTLC (High-Performance Thin Layer Chromatography) perform as an effective method to measure by the quality and quantity of *Amalaki Avaleha*. Standard reference and mobile phase are used for HPTLC profiling of both ascorbic acid and Gallic acid. The phytochemical in *Amalaki Avaleha* is verified by the identification of clear bands in the sample lane at Rf values matching those of the standard marker. After that for observing bands, UV and Visible light are used. The ascorbic acid present in *Amalaki Avaleha* is used as antioxidant, Cardioprotective, Hepatoprotective, Antidiabetic, Anticancer activity. As well as gallic acid, is a type of phenolic compound, is understand for its antimicrobial properties, cardiovascular benefits, gastrointestinal effect, metabolic activities, neuropsychological impact which enhance the formulation's therapeutic efficacy. Clinical research which had done on patients of anaemia got good results, the symptoms presented by patients has reduced. The study also emphasizes the significance of standardized processing techniques and the careful selection of raw materials to preserve the medicinal quality of Ayurvedic preparations. HPTLC use for such standardization ensures a reproducible, reasonable and effective scientific method that connects traditional approaches with contemporary scientific verification.

CONCLUSION

The significance of HPTLC analysis in *Amalaki Avaleha* lies in determining the levels of Ascorbic acid and gallic acid. Ascorbic acid plays a crucial role in enhancing iron absorption. Improved iron absorption leads to a reduction in iron levels and haemoglobin deficiency in the body, which helps to relieve anaemia. As a result, the related symptoms of anaemia may diminish. In children, anaemia frequently arises from causes such as malnutrition, the intake of non-nutritive substances (like soil or clay), hookworm infestations and the early introduction of cow's milk. Additionally, ascorbic acid is acknowledged as one of the most potent antioxidants and aids in supporting the immune system. The aim of HPTLC help as to estimate the quality, consistency, safety and reliability of *Amalaki Avaleha*.

CONFLICT OF INTEREST

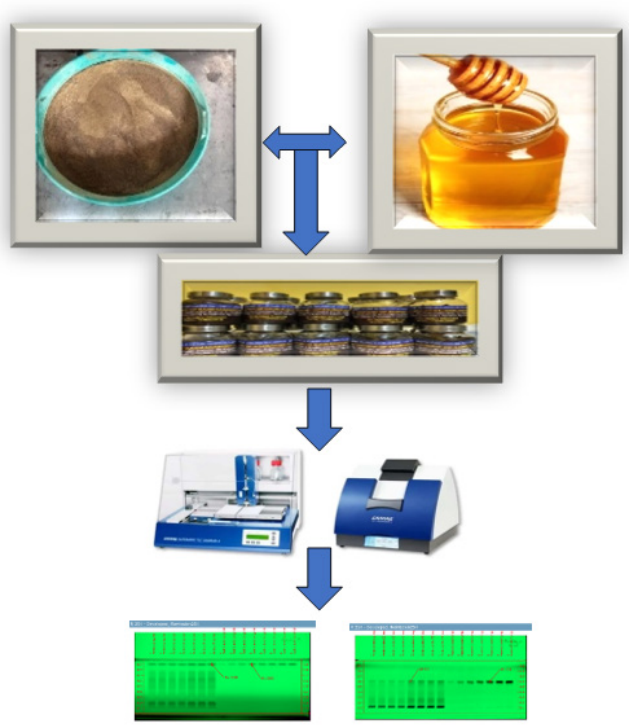
The authors declare that there is no conflict of interest.

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GRAPHICAL ABSTRACT



SUMMARY

This study emphasizes *Amalaki Avaleha*, a traditional Ayurvedic remedy cited in the Sushruta Samhita for addressing Pandu Roga (anemia). Given that its therapeutic benefits largely hinge on the presence of Gallic acid and Ascorbic acid, the research employs High-Performance Thin Layer Chromatography (HPTLC) to scientifically verify and confirm their existence. By standardizing these essential phytochemicals, the research guarantees that *Amalaki Avaleha* maintains consistent quality, is safe for use, and delivers its medicinal advantages effectively.

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