ORIGINAL ARTICLE

High-Performance Thin-Layer Chromatography Analysis of Gallic Acid and Other Phytoconstituents of Methanolic Extracts of *Myrica nagi* Fruit

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ABSTRACT

Background: *Myrica nagi* Thunb. (family: *Myricaceae*) is effective against gastric, metabolic, and hepatic disorders. The therapeutic effect of its fruit, which is consumed in North India, has not been confirmed, and detailed chemical profiling of the fruit is thus required. **Objectives:** The study objective was to develop and optimize a high-performance thin-layer chromatography (HPTLC) method for the characterization of gallic acid, quercetin, myricetin, and caffeic acid in the methanolic extract of *M. nagi* fruit and the quantification of gallic acid. **Materials and Methods:** Analyses were performed using HPTLC, and liquid chromatography-mass spectrometry. HPTLC experiments were carried out using an optimized solvent mixture, which enabled the separation and detection (at 254 and 366 nm) of four flavonoid compounds in the dried *M. nagi* extract. Gallic acid was quantified using calibration curves. **Results:** The proposed method enabled the detection of gallic acid, quercetin, myricetin, and caffeic acid. Validation took into account the estimation of linearity, limit of detection, limit of quantification, accuracy, and recovery of gallic acid. Gallic acid was quantified at 12.93 µg/mg of dry plant concentrate. **Conclusion:** This study describes the development of an HPTLC method for the analysis and characterization of phytoconstitutents in the methanolic solution of a dried *M. nagi* fruit extract. The method was successfully validated for the analysis of gallic acid.

Key words: Flavonoid, gallic acid, high-performance thin-layer chromatography, liquid chromatography-mass spectrometry, *Myrica nagi*

SUMMARY

We performed full chemical profiling of the fruit extract of *Myrica nagi* and provided insights into the various phytoconstituents present in the fruit through LC-MS. The presence of various phytocompounds was confirmed through HPTLC. The method for gallic acid analysis was validated in a solvent system of ethyl formate/toluene/formic acid/water 20:1:2.6:0.5 (*v/v/v/v*). The method allowed excellent separation of the compounds and can be of high importance for further quantification of phytocompounds and for the development of herbal formulations.
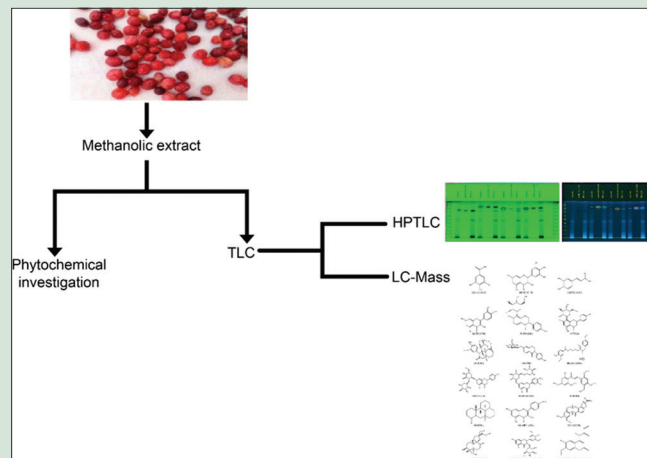
Abbreviations Used: HPLC: High-pressure liquid chromatography, HPTLC: High-performance thin-layer chromatography, ESI: Electrospray ionization, LC-MS: Liquid chromatography-mass spectrometry, LOD: Limit of detection, LOQ: Limit of quantification, MS: Mass spectrometry, Rf: Retention factor, TLC: Thin-layer chromatography.

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INTRODUCTION

Myrica nagi Thunb. (syn. *Myrica esculenta*), commonly known as box myrtle, katphala, boxberry, and orkaphal, belongs to the family *Myricaceae* and is a widely used medicinal plant. *M. nagi* is sourced primarily for its fruits,[1] which are similar to raspberries and are deep red in color, with a small amount of pulp and a round seed at the center. The bark of this plant contains chemical compounds, such as myricetin, myricitrin, and specific glycosides. It has been reported to possess anti-allergic,[2] anti-inflammatory,[3] antioxidant,[4] antihelminthic,[5] antimicrobial,[6] anxiolytic,[7] chemoprotective,[8] hypertensive,[9] mast cell-stabilizing,[10] analgesic,[11] antiulcerating,[12] antidiabetic,[13] hepatoprotective,[14] and wound-healing[15] properties. This has led to the development of new therapeutic medicines using *M. nagi* extract.[16]

Analysis of herbal samples typically involves several steps, including sample preparation, separation, and detection. Sample preparation is the first essential step and involves the extraction of analytes of interest. Modern, state-of-the-art sample preparation and separation methods offer substantial benefits over standard methods for the characterization of medicinal plants, playing an essential role in the commercialization of high-quality herbal products.[17] Thin-layer chromatography (TLC) is a simple and cost-effective separation technique enabling the analysis

of several samples simultaneously; hence, it is often considered the first option for diverse medicinal analytical applications. High-pressure liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) are widely employed to characterize and analyze numerous bioactive mixtures, enabling the detection of constituents, including secondary metabolites, nutrients, and amino acids.[18] Hence, HPTLC represents an attractive technique for qualitative and quantitative analyses of herbal extracts.[19] Mass spectrometry (MS) provides highly accurate structural information about the constituents of herbal extracts, allowing the identification of compounds of interest. Overall, an effective, high-throughput screening and separation technique combined with MS detection is crucial for the identification of potential natural therapeutic candidates.[20]

This study aimed to develop and validate a high-throughput HPTLC method for the characterization of gallic acid, quercetin, myricetin, and caffeic acid in methanolic extracts of the *M. nagi* fruit. The developed method was validated for gallic acid.

MATERIALS AND METHODS

Chemicals

All solvents, including methanol, butanol, acetic acid, formic acid, and toluene, were of analytical grade and purchased from SD Fine Chemicals (Mumbai, Maharashtra, India). Flavonoid standards, such as those for gallic acid, quercetin, myricetin, and caffeic acid, were purchased from Natural Remedies (Bengaluru, Karnataka, India).

Collection of *Myrica nagi*

Fruit samples of *M. nagi* were collected from Mandi district, Himachal Pradesh, India, in July 2017. The collected plant material was authenticated by NISCAIR, Delhi, India (Ref. No. NISCAIR/RHMD/Consult//2017/3102-51-4). Healthy fruits were separated and kept for further analyses.

Methanolic extraction of *Myrica nagi* fruits

Freshly collected *M. nagi* fruits were dried in the shade and milled into a coarse powder. Then, 500 g of the finely powdered sample was subjected to a 4-h extraction with methanol at 55°C–65°C using a Soxhlet device (Buchi, Mumbai, Maharashtra, India). Before and after each extraction, the organic extract was accurately weighed, dried, and passed through a filter paper of 120 mm diameter and medium porosity (S and S., Dassel, Germany). This extraction step was repeated thrice, and the extract was eventually dried using a rotary evaporator (Rotavapor Heizdab WB eco, Heidolph Instruments, Schwabach, Germany). The dried extracts were combined prior to further analyses.

Preliminary phytochemical investigation

The extract was subjected to phytochemical screening following standard methods.[21,22]

Preparation of sample and standard solutions

Standard stock solutions of 1 mg/mL were prepared by dissolving the standards in methanol. Standard stock solutions were further diluted in methanol to obtain a working solution of 25 μg/mL. An appropriate amount of weighed methanolic extract was dissolved in methanol to obtain a 10 mg/mL sample solution.

High-performance thin-layer chromatography analysis

Precoated silica gel 60F$_{254}$ TLC plates were first washed with methanol. The silica was activated by placing the plates in an oven at 60°C for 15 min. The solutions were applied to the plates using an automatic TLC sampler (CAMAG® ATS; CAMAG, Muttenz, Switzerland) equipped with a 100-μL syringe at a steady implementation rate of 150 nL/s. The solutions were applied onto prewashed TLC plates in 5-mm broad bands, 10 mm from the bottom edge, 10 mm from the side and top edges, and with 6 mm gaps between each spot. The CAMAG® Twin Trough Chamber was presaturated for 20 min at 25°C ± 2°C and 40% relative humidity, with the mobile phase composed of a mixture of ethyl formate/toluene/formic acid/water 20:1:2.6:0.5 (v/v/v/v).[23-25] The chromatographic run was approximately 80 mm. After separation, the TLC plates were dried using an air current. Densitometric visualization of the chromatographic spot was performed at 254 nm and 366 nm using deuterium and mercury lamps in the CAMAG® HPTLC instrument equipped with visionCATS software (CAMAG, Muttenz, Switzerland).

Characterization of methanolic extracts using liquid chromatography–mass spectrometry

The methanolic extract was analyzed using the 2795 Alliance HPLC system (Waters, Milford, MA, USA) coupled with a Micromass Q-TOF Micro Mass Spectrometer (Waters, Milford, MA, USA). Separation was carried out on an XBridge C$_{18}$ column (130 Å, 3.5 μm, 4.6 mm × 150 mm; Waters). The mobile phase was composed of 80% methanol and 20% water and run in the isocratic mode. The flow rate was set at 0.7 mL/min. The injection volume was 20 μL. MS acquisition was performed using the electrospray ionization (ESI)-positive mode and multiple reaction monitoring with unit resolution. Desolvation gas and cone gas flow rates were set to 550 L/h and 30 L/h, respectively. Desolvation gas and source temperatures were set to 300°C and 110°C, respectively. The ESI capillary voltage was fixed at 3000 V, and the cone voltage was set at 30 V. Compounds were fragmented using collision energy of 4 eV. Nitrogen and argon were used at pressures of 6–7 bars and 5–6 bars, respectively.

Validation of the developed high-performance thin-layer chromatography method

The developed HPTLC method was validated for the quantification of gallic acid in terms of specificity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) following the ICH guidelines.[26,27]

Precision

Intra- and inter-day precision was evaluated using a standard solution of gallic acid at 100 ng/spot. Solutions were injected 12 consecutive times (intraday precision) on 2 consecutive days (interday precision). The results were evaluated using retention factor (Rf), peak area, and standard deviation.

Limit of detection and limit of quantification

The LOD and LOQ were calculated using the gallic acid calibration curve. Different volumes of gallic acid solution (1.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 μL) were applied to the plate, together with different volumes of the methanolic extract (2.0, 2.0, 3.0, 3.0, 4.0, and 4.0 μL).

Accuracy

Accuracy reflects the positive and negative influences of other components present in the mixture on the quantification. The standard addition method was used to determine the accuracy of the developed protocol by calculating recoveries. Known and varying amounts of gallic acid were added at three different concentrations (80%, 100%, and 120%) of a predetermined amount. The average recovery and total recovery of gallic acid were calculated and expressed in percentages.

Specificity

The specificity of the developed method was evaluated by measuring standard solutions, blank samples, and test samples. The spot of gallic acid obtained from the analysis of test samples was confirmed by comparing the R_f values and spectra of the standard solutions. The purity of gallic acid was evaluated by observing the spectral peak's start, apex, and end for the spot.

RESULTS

This study aimed to identify flavonoids, gallic acid, quercetin, myricetin, and caffeic acid in methanolic extracts of the *M. nagi* fruit using HPTLC. These flavonoids have been shown to be among the active components of other herbal extracts. HPTLC was used for the qualitative and quantitative analyses of gallic acid.

Extraction yield and phytochemical analysis

Five hundred grams of the powdered fruit yielded 8.83 g of extract. The extract was a semi-solid, dark-brown mass with a characteristic odor. It was positive for the presence of alkaloids, glycosides, flavonoids, steroids, amino acids, carbohydrates, tannins, and phenols.

High Performance Thin-layer chromatography analysis of *Myrica nagi* extract

Table 1 presents the results of HPTLC analysis of the methanolic extract. Separation of the extract into single components is shown in Figures 1 and 2. The extract produced identical spots to those obtained for the standard samples. Gallic acid, was further quantified and new method was validated.
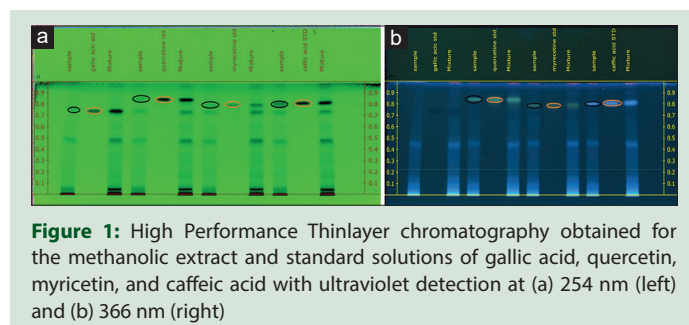

Figure 1: High Performance Thinlayer chromatography obtained for the methanolic extract and standard solutions of gallic acid, quercetin, myricetin, and caffeic acid with ultraviolet detection at (a) 254 nm (left) and (b) 366 nm (right)

Liquid chromatography-mass spectrometry analysis of *Myrica nagi* extracts

The LC-MS analysis of the methanolic extract revealed 23 compounds, of which 18 were confirmed using the European MassBank database. The identified compounds included gallic acid, myricetin, caffeic acid, quercetin, puerarin, vitexin, brucine, mebeverine, ononin, rhoifolin, hesperidin, matrine, rotenone, rubone, syringetin-3-*O*-galactoside, lagochilline, 7-chloro-4-methyl-2-oxochromen-6-yl propanoate, and kaempferide. The structures of these compounds are presented in Figure 3. The obtained mass spectrum is shown in Figure 4. Table 2 lists the detected compounds.

Validation of the newly developed high-performance thin-layer chromatography method for the quantification of gallic acid

The method was validated and assessed for precision, accuracy, specificity, LOD, and LOQ using ICH guidelines.[26]
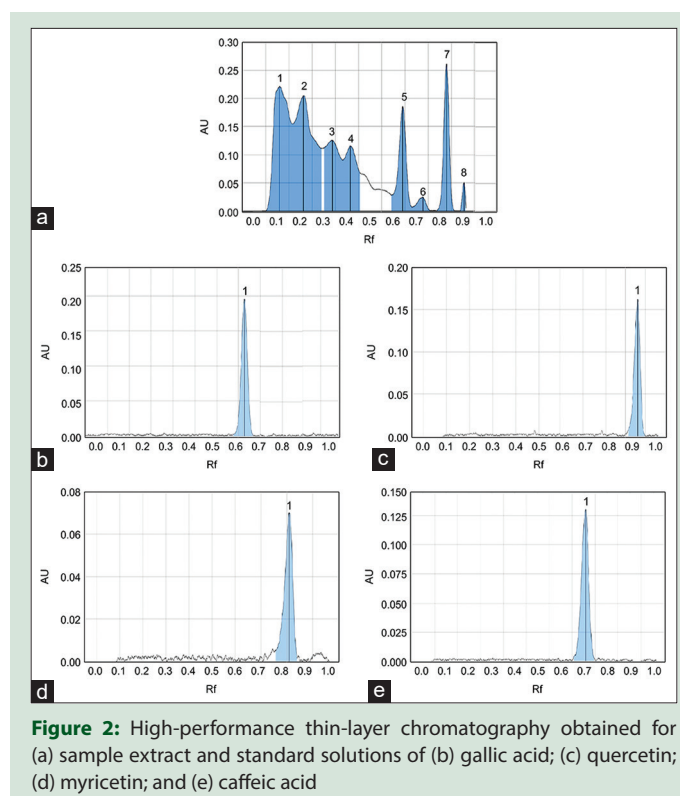

Figure 2: High-performance thin-layer chromatography obtained for (a) sample extract and standard solutions of (b) gallic acid; (c) quercetin; (d) myricetin; and (e) caffeic acid

Table 1: Retention factors of the different peaks observed in the methanolic extract of *Myrica nagi* by high-performance thin-layer chromatography analysis

| Peak	Start		Maximum			End		Area (%)	Manual peak	Substance
	RF	Height	RF	Height	Percentage	RF	Height			
1	0.100	0.0000	0.159	0.2210	18.62	0.216	0.1538	0.01757 (25.35)	No	Unknown
2	0216	0.1538	0.262	0.2047	17.25	0.342	0.1109	0.01952 (28.17)	No	Unknown
3	0.350	0.1116	0.388	0.1250	10.53	0.430	0.0900	0.00906 (13.08)	No	Unknown
4	0.430	0.0900	0.465	0.1150	9.69	0.506	0.0670	0.00738 (10.65)	No	Unknown
5	0.642	0.0276	0.690	0.1855	30.63	0.731	0.0062	0.00707 (30.20)	No	Gallic acid
6	0.731	0.0062	0.778	0.0240	25.02	0.810	0.0004	0.00105 (25.52)	No	Quercetin
7	0.833	0.0000	0.879	0.2615	22.03	0.914	0.0000	0.00694 (22.02)	No	Myricetin
8	0.938	0.0000	0.955	0.0501	20.22	0.963	0.0048	0.00071 (20.02)	No	Caffeic acid
9	0.706	0.0032	0.757	0.1302	100	0.795	0.0028	0.00491 (100)	No	Gallic acid standard
10	0.811	0.0069	0.860	0.1628	100	0.896	0.0013	0.00557 (100)	No	Quercetin standard
11	0.758	0.0051	0.809	0.0703	100	0.840	0.0016	0.00261 (100)	No	Myricetin standard
12	0.776	0.0022	0.827	0.1960	100	0.870	0.0003	0.00666 (100)	No	Caffeic acid standard

RF: Retention factor
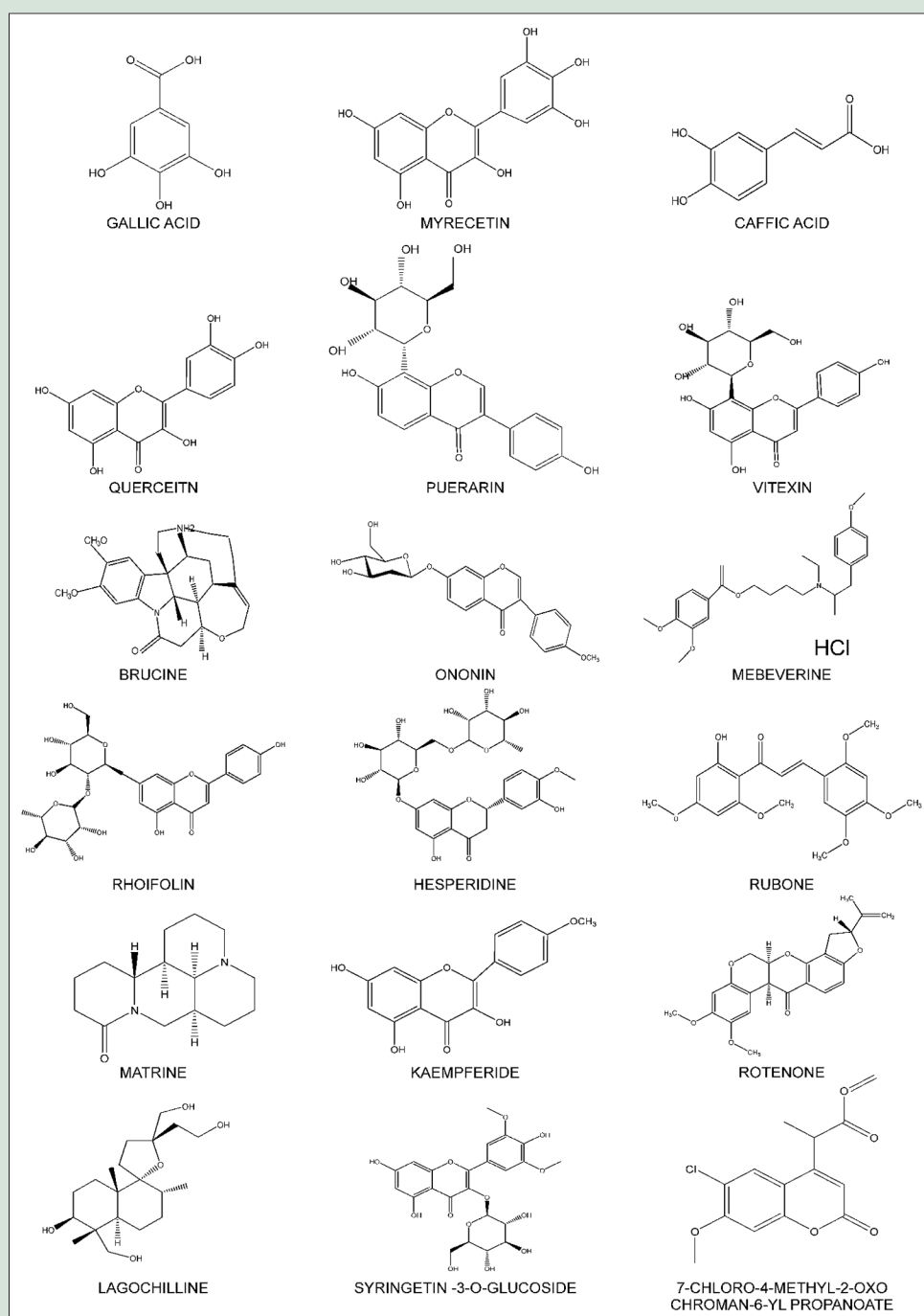
Figure 3: Structure of different compounds detected in *Myrica nagi* fruit extract by liquid chromatography-mass spectrometry

Figure 5 shows the results of precision evaluation, including intra- and inter-day repeatability. Figure 6 and Table 3 show the results of LOD and LOQ estimation. The evaluation of accuracy and recovery is shown in Figure 7 and Tables 4 and 5. Finally, specificity evaluation results are shown in Figure 8; they clearly demonstrated that bands were not observed in the mobile phase or in the solvent.

Table 6 presents a summary of the results for the various parameters evaluated during method validation. Linearity ranged from 100 to 700 µg/spot, using a regression equation of $y = 1.636 \times 10^{-8} \times - 1.904 \times 10^{-4}$. The accuracy/recovery of gallic acid was 90%–110%.

Furthermore, this method exhibited a high precision, with a coefficient of variation of 1.65%, which was comfortably below the 3% tolerance limit. The LOD was 38.3 ng and the LOQ was 116.1 ng. The proposed method was determined to be highly specific due to the absence of bands in the mobile phase and solvent front. The method was finally employed to quantify the amount of gallic acid present in the methanolic extract, which was determined to be 129.3 µg/10 mg of dry plant extract.

DISCUSSION

In this study, we first evaluated the phytochemical parameters of

Table 2: List of compounds detected in the methanolic extract of *Myrica nagi* by liquid chromatography-mass spectrometry and identified based on data from the European MassBank database

Peak number	RT	Peak height	Peak area	Area (%)	m/z	Compounds	Reference
1	0.48	10900	5453.11	2.77	113	Unknown	European MassBank
2	0.97	1599	7050.40	3.59	114	Unknown	European MassBank
3	2.40	16111	12757.56	20.49	170	Gallic acid	European MassBank
4	2.57	14768	4902.14	2.49	266	7-chloro-4-methyl-2-oxochromen-6-yl propanoate	European MassBank
5	3.13	7126	1391.96	20.71	180	Caffeic acid	European MassBank
6	3.60	7766	3003.83	1.53	248	Matrine	European MassBank, PubChem
7	4.20	6757	4018.11	2.04	356	Lagochilline	European MassBank
8	7.57	9315	5901.00	30.00	301 302 318	Quercetin, myricetin	European MassBank
9	8.37	10825	6278.49	3.19	363	Unknown	European MassBank, NIST
10	8.82	10268	3740.68	1.90	432	Vitexin	European MassBank
11	9.10	7495	1267.57	0.64	394 394	Brucine, rotenone	European MassBank, PubChem
12	9.62	12354	3492.32	1.78	374.4	Rubone	European MassBank
13	10.58	104809	63657.38	32.38	416.3 429.6	Puerarin, mebeverine	European MassBank
14	14.32	4678	2315.99	1.18	300 430	Kaempferide, ononin	European MassBank
15	22.95	5551	2091.33	1.06	610.5	Hesperidin	European MassBank
16	23.85	2595	687.74	0.35	508	Unknown	European MassBank
17	25.08	2352	703.16	0.36	509	Syringetin-3-*O*-galactoside	European MassBank
18	26.93	0	2691.22	1.37	515	Unknown	European MassBank
19	27.73	13111	3912.74	1.99	551	Unknown	European MassBank
20	28.28	83122	37248.86	18.95	578.52	Rhoifolin	European MassBank
21	31.07	20358	12272.02	6.24	644	Unknown	European MassBank
22	31.23	18942	5375.77	2.73	645	Unknown	European MassBank
23	31.60	13779	6369.86	3.24	693	Unknown	European MassBank

RT: Retention time

Table 3: Limit of detection and limit of quantification for gallic acid

Concentration (μg/mL)	Area	SD	LOD (ng)	LOQ (ng)
100	0.001462	0.00019	38.3	116.1
200	0.003085			
500	0.007783			
600	0.009924			
700	0.011149			

UV detection was carried out at 254 nm. LOD: Limit of detection; LOQ: Limit of quantification; SD: Standard deviation; UV: Ultraviolet
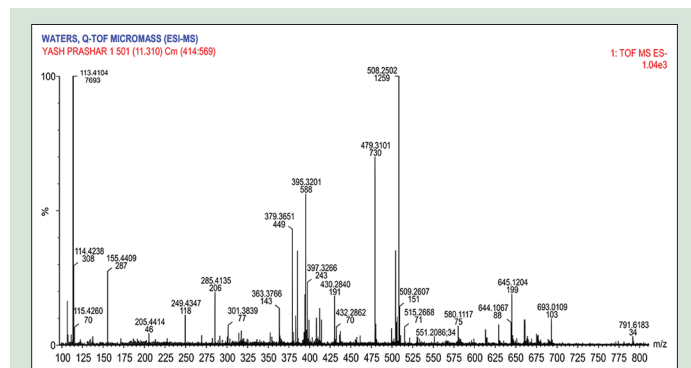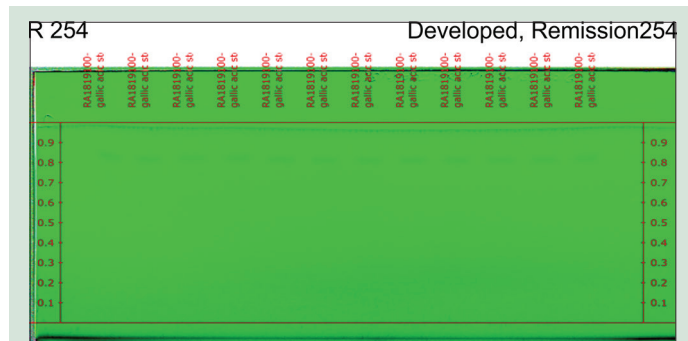

Figure 4: Mass spectrum of *Myrica nagi* fruit extract

a methanolic extract of the *M. nagi* fruit using HPTLC analysis. Because the antioxidant potential of *M. nagi* is influenced by its polyphenolic content,[28] it is essential to investigate the bioactivity of each detectable polyphenolic compound present in the fruit of *M. nagi*. The use of flavonoids as therapeutics against various diseases has gained momentum in recent years.[29] Phytoconstituents of *M. nagi* identified previously through LC-MS and HPTLC analyses have been demonstrated to act against tumors, asthma, chronic bronchitis, and several digestive and endocrine disorders.[30] The newly developed HPTLC method enabled the analysis and quantification of gallic acid, a potential antioxidant[31] present in this plant. Previous HPTLC studies performed on other plants had reported an R_f value of 0.57 for gallic acid,[32] which corresponds to the value obtained here. *M. nagi* grows in forests and is used by the local Himalayans (Uttarakhand and Himachal Pradesh). Gallic acid, which is known for its potent antioxidant activity, has not been detected before in the fruit of this species. By quantifying gallic acid and confirming the presence of various phytocompounds in the *M. nagi* extract via HPTLC and LC-MS, our study offers new information aimed at the use of this plant for therapeutic purposes and even industrial applications. Several studies have described the development of HPTLC methods for the quantification of compounds such as myricetin in the stem bark of *M. esculenta*.[28] Our method is advantageous as it enables the future quantification of several other identified compounds, such as quercetin, myricetin, and caffeic acid. The reported R_f values for quercetin (0.98), myricetin (0.53),[28] and caffeic acid (0.78)[32] are identical to the ones obtained in our study. This newly developed strategy will prove essential for the standardization of methods associated with identifying and quantifying compound in extracts from this plant, many of which are used in various polyherbal formulations. HPTLC is a common method for characterizing complex herbal mixtures due to its elevated resolution of phytochemicals, which allows for precise and accurate quantitative analysis.[33]

Table 4: Recovery of gallic acid

Sample	Mass of gallic acid present in the sample (ng)	Mass of gallic acid (ng)	Total calculated mass of gallic acid (ng)	Detected mass of gallic acid (ng)	Recovery (%)
1	170.16	90.75	260.91	272	104.8
2	170.16	113.44	283.6	286	101.3
3	170.16	136.12	306.28	336	109.5

Figure 5: High Performance Thinlayer chromatography plate for precision (i.e., intraand interday variabilities). Ultraviolet detection performed at 254 nm
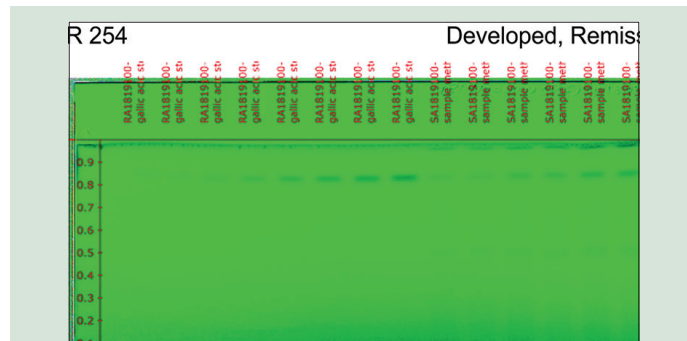
Figure 6: High Performance Thinlayer chromatography plate for limit of detection and limit of quantification. Ultraviolet detection carried out at 254 nm
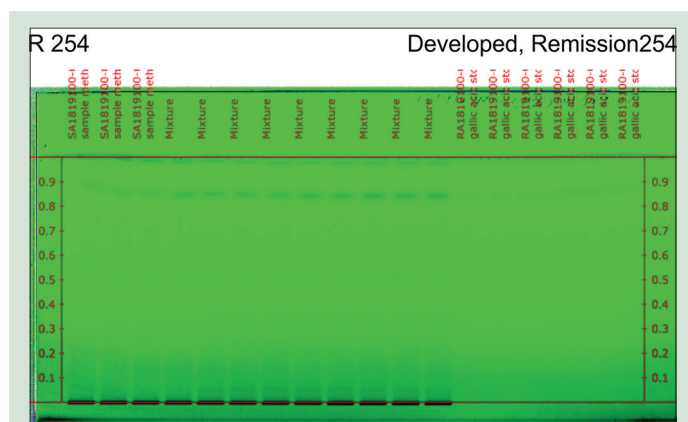
Figure 7: High Performance Thinlayer chromatography plate for accuracy. Ultraviolet detection carried out at 254 nm
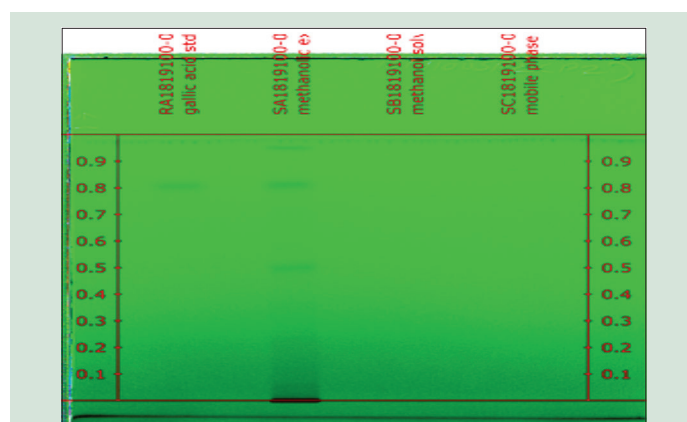
Figure 8: High Performance Thinlayer chromatography plate for specificity. Ultraviolet detection carried out at 254 nm

In this study, the HPTLC method was optimized to obtain a characteristic profile of the *M. nagi* fruit. The selected mobile phase provided comparable Rf values to standard compounds, demonstrating sufficient resolution and specificity by applying a single and fast run. With very high concentrations and using UV detection at 254 nm, eight compounds were detected in the fruit extract. Furthermore, various experimental factors associated with this protocol, such as sample volume and LOD, had to be optimized. For example, the optimized sample volume was found to be 3 µL, which provided reproducible and accurate profiling, as well as information related to the number of compounds present in the extract, while facilitating the equilibrium between the various categories of phytocompounds present in the extract.

CONCLUSION

The developed and validated HPTLC method represents an effective technique for the quantification of gallic acid in a methanolic solution of *M. nagi* extract. The obtained results show that the methanolic extract of *M. nagi* contains various active compounds that may account for its therapeutic properties. Specifically, this study highlights the important therapeutic phytoconstituents of the fruit of *M. nagi*, which have been neglected by previous investigations. Due to the short shelf life of the fruit, the phytoconstituents contained in it need to be extracted as soon as the fruit ripens, for maximum benefit. Our study also suggests the use of *M. nagi* in herbal formulations for conditions related to stomach and endocrine disorders as the phytoconstituents identified here have been reported to confer such benefits. Furthermore, the proposed HPTLC method proved to be specific, accurate, and precise and was characterized by a high recovery rate. Additional phytoconstituents of the methanolic extract of *M. nagi* are currently under evaluation.

Table 5: Accuracy of gallic acid recovery values

Expected value	Percentage	Mean (%)
0.009667869	104.8	
0.01039752	101.3	105.2
0.009975397	109.5	

Table 6: Summary of the validation parameters estimated using the developed high-performance thin-layer chromatography method

Parameter	Results
Linearity (quantification)	100-700 µg/spot
Linear regression equation	$y = 1.636 \times 10^{-8}x - 1.904 \times 10^{-4}$
Accuracy (recovery of gallic acid) (%)	90-110
Precision (CV) (%)	1.65
Interday (%)	1.4
Intraday (%)	1.6
LOD (ng)	38.3
LOQ (ng)	116.1
Specificity	Specific

CV: Coefficient of variation; LOD: Limit of detection; LOQ: Limit of quantification

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

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