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Original Article

Marker Based Standardization of Himalayan Berry: *Myrica esculenta* Buch.-Ham ex D. Don

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ABSTRACT

Background: *Myrica esculenta* Buch.-Ham ex D. Don (Syn-*M. nagi*) commonly known as Himalayan berry/ *Kaphal*/ Katphal is an economically important medicinal plant with multipurpose uses. The medium sized tree is utilized for its bark, flowers, roots and fruits in Ayurvedic and Unani system of medicine due to its therapeutic potential. The fruits of *M. esculenta* are sold in local markets as jam, squashes, pickles etc and are also consumed by the local populations. However, the therapeutic properties of the fruits remain largely ignored. **Objectives:** The aim of this study is to develop standard quality control parameters and standardize the extracts of fruits of *M. esculenta* in terms of bioactive marker content and give it a recognition of a standardized extract before it becomes part of a herbal formulation. **Materials and Methods:** Standardization of fruits of *M. esculenta* has been carried out in terms of its pharmacognostic evaluation - physicochemical and phytochemical analysis. Chromatographic separation was achieved to develop the best resolved HPTLC and HPLC fingerprints as a quality control tool. Gallic acid, a bioactive marker, was quantitated from *M. esculenta* fruits collected from various regions of Uttarakhand, different morphological parts and from marketed formulation using validated HPTLC and HPLC method. **Results:** The LOD and LOQ levels were found to be 8 and 10µg/mL for HPTLC and 0.25 and 0.50µg/mL for HPLC respectively with a linear response range of 10-500 µg/mL for HPTLC and 0.50-50µg/mL for HPLC. The (r^2) was found to be greater than 0.99 using both techniques. The concentration of phytochemical marker varied in samples collected from different regions of Uttarakhand. Variation in the marker content has been also observed from different morphological parts and in marketed formulation. **Conclusion:** To attain reproducible quality of herbal products selection and use of authentic plant material is of utmost need. Unique fingerprints can be used as a quality control tool for the use of authentic sample of *M. esculenta* singularly and as a part of various medicinal formulations. The method developed for the estimation of gallic acid can be applied to matrices containing *M. esculenta* fruits. The findings of the study will enable sustainable harvest of *M. esculenta* fruits and its processing as a value-added minor forest produce of Garhwal Himalayas.

Key words: Fruits, Gallic acid, HPTLC, HPLC, *Myrica esculenta*, Validation.

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INTRODUCTION

Myrica esculenta Buch.-Ham ex D. Don (Syn- *M. nagi*, Myricaceae), commonly known as Himalayan berry and locally known as Kaphal is a tree which is revered by locals for its delicious ripe fruits, especially in Uttarakhand, North India. It is a sub-temperate evergreen tree distributed in the mid-Himalayan regions between 1300 meters and 2100 meters, spanning from Pakistan, India, Nepal and China.[1] Traditionally the fruit of *M. esculenta* is relished by local communities and is also made into pickles and drinks. Although its medicinal properties remain largely ignored by the local population, the tree is highly valued for its medicinal uses in Ayurvedic and Unani systems of medicine. Its bark, flowers, fruits and leaves are used against various ailments including mennorhagia, asthama, anaemia, tumors, bronchitis, menstrual disorders etc.[2-5] *M. esculenta* is reported for its hepatoprotective, antibacterial, antifungal, anti-helminthic and anti-inflammatory activities.[6-12] Due to its restricted geographical distribution, seasonal availability and short shelf-life, much produce is locally consumed, with very little commercial harvesting.

The plant is reported to be rich in various bioactive phytochemicals including gallic acid, ellagic acid, catechin, myricetin, stigmasterol, beta sitosterol, lupeol, quercetin etc.[12-14] The fruit is also rich in amino acids, ascorbic acid, caffeic acid, trans-cinnamic acid etc. Owing to its varied medicinal application, *M. esculenta* is an important ingredient of various Ayurvedic formulations including *Chawyanprash, Katphaladi churna*, *Khadiradi gutika* and *Pushyanuga churna* etc.[4,12,15,16]

To ensure therapeutic efficacy of a medicinal plant, it is important to maintain the right time of collection, the appropriate method of harvesting and the correct selection of the site of harvest while collecting authenticated plant part in order to benefit from its therapeutic potency.[17-19] Validation of these collection factors are generally ignored during commercial exploitation and therefore the crude plant raw material may get substituted or adulterated with other low quality and morphologically similar medicinal plants which tend to reduce the therapeutic efficacy.[20,21] *M. esculenta* has tremendous therapeutic potential, with every part of the tree being used medicinally. In view of its pharmacological importance in traditional and modern systems of medicine it is necessary to develop validated quality standards for *M. esculenta*. The bark of *M. esculenta* is widely used in traditional and modern systems of medicine but the data on fruits for their phytochemical content and validated chromatographic evaluation is scant. Methanolic extracts of the fruits have been evaluated for their phytochemical constituents using HPTLC techniques but the study is reported on a single location collection.[22]

In the current work, phytochemical fingerprints of ethanolic extract of fruits of *M. esculenta* have been developed using HPTLC and HPLC techniques. These techniques have been validated and applied for the quantification of the bioactive marker gallic acid. Gallic acid is a biologically active phytochemical marker, a phenolic compound, reported to have anti-inflammatory, anticancer, hepatoprotective, anti-oxidant activities.[23,24] To generate standards for quality related parameters, different plant parts, unripe and ripe fruits, fruits collected from different regions of Garhwal Himalayas have been compared using validated HPTLC and HPLC techniques. Further, the application of these validated chromatographic techniques has been demonstrated in the estimation of gallic acid from an Ayurvedic medicinal formulation, *Pushyanuga churna* which uses *M. esculenta* as an ingredient.

MATERIALS AND METHODS

Apparatus

- HPTLC

Spotting device of CAMAG Linomat V Automatic Sample Spotter (Muttenz, Switzerland) was used for spotting the plate. Syringe of 100 µL (Hamilton) was used for spotting the samples on HPTLC plates of 20 × 10 cm dimension precoated with silica gel 60 F_{254} (0.2 mm thickness; Batch. No. HX50034254; Merck, Darmstadt, Germany).TLC chamber of CAMAG glass twin trough chamber (20 × 10 × 4 cm) dimension was used for plate development. Densitometer of CAMAG TLC Scanner 4 linked to WINCATS software and Photo-documentation system of CAMAG Reprostar 3 was used for scanning and photo documenting the plate during the study.

- HPLC

HPLC Cosmosil C_{18} column (150 × 4.6 mm, 5.0 µm) of Batch. No. K66323 was used for separation of analytes. HPLC system of (JASCO) comprising of binary pumps (PU 1580) with rheodyne injector (20 µL loop) and Photodiode array detector (PDAD; MD-1510) was used throughout the analysis.

Chemicals and Reagents

All chemicals and reagents used have been purchased as Analytical Grade and HPLC grade from Merck (India) Limited. The standard of Gallic acid was procured from Sigma Aldrich (purity ≥95%, Batch No. SLBM9643V).

Samples

(a) Plant materials- *M. esculenta* fruits were collected from different regions of Uttarakhand, (India) in the month of May every year between 2016-2019. The plant materials were authenticated by CSIR-National Botanical Research Institute, Lucknow (Authentication No. LWG103078). The fruits and other plant parts were shade dried for 14 days, then dried at 37±2°C for a week, powdered in a mixer grinder, sieved through BSS 85mesh (Jayant Scientific Industries, Mumbai, India) to obtain fine powder and was stored in air-tight containers that were used for further analysis.

Preparation of Standard Solution

A 10mg mass of the standard (gallic acid) was accurately weighed and transferred to methanol (10.0 mL) in an amber-colored standard volumetric flask. The content was initially dissolved in a minimum quantity of methanol, sonicated and then diluted with methanol. The stock solution of 1000.0µg/mL was used to prepare working standard solutions of 100.0µg/mL, 10.0µg/mL and 1.0 µg/mL respectively.

Preparation of Calibration Curve and Quality Control Samples

A seven-point linear calibration curve was obtained by preparing appropriate dilutions of standard gallic acid. The working standard concentration for gallic acid was in the range of 10.0–500.0 µg/mL for HPTLC and 0.5–50.0 µg/mL for HPLC. Further, low, middle and high concentration levels of quality control samples (LQC, MQC and HQC) were prepared for precision, accuracy and robustness experiments while validating the developed chromatographic methods.

Preparation of Sample Solutions

Assay: The ethanolic extract (10 mg) of *M. esculenta* fruits and other plant parts were dissolved in ethanol (1.0 mL), vortexed for a minute and sonicated for 20 min. The solution was further filtered through a nylon micro filter paper (0.45 µm) and was subjected to HPTLC and HPLC analysis for development of a phytochemical fingerprint and for separation and quantitation of gallic acid.

Method Application

The powder of *Pushyanga churna* (0.5g) was dissolved in (5.0) mL of ethanol, vortex mixed for a minute and sonicated for 20 min. This was passed through a nylon micro filter paper (0.45 µm) and was subjected to HPTLC and HPLC analysis.

EVALUATION OF QUALITY CONTROL PARAMETERS

Physicochemical and Phytochemical Evaluation

The physicochemical parameters of the *M. esculenta* (fruit) including foreign organic matter, loss on drying, ash content (total, acid insoluble and water soluble) and extractive values were determined using standard pharmacopoeia methods.[4,25,26] Similarly, the qualitative phytochemical screening of some major class of secondary metabolites (flavonoids, tannins, glycosides, alkaloids and resins) was carried out by performing preliminary phytochemical test as per reported methods.[26] Results are tabulated in Table 1 and Table 2.

Macroscopic evaluation[1,4,27]

Macroscopic evaluation of fruits and other plant parts of *M. esculenta* were carried out and the characteristics matched reported features as listed below;

Fruit: A drupe, ellipsoid or ovoid, 0.7-1.0 cm long, 0.5-0.7 cm wide, surface tubercled, very hard. Taste is sourish when unripe and is usually green it is sweet when fully ripe and is dark red in color.

Leaves: Lanceolate with entire or serrate margins, pale green at lower surface and dark green at the upper surface; it is usually 9-12cm in length and 3-3.5cm in width and is mostly crowded towards the end of branches.

Bark: Usually 12-15m in height with trunk diameter about 92.5 cm. Outer bark is grayish dark in color, rough vertically wrinkled, white inner bark is dark brown in color with smooth surface.

Seed: Ovoid, 0.6 cm long, 0.3 cm wide, surface very smooth, light brown; taste, oily.

The macroscopical features such as shape, texture, size, colour and taste of the fruit of *M. esculenta* are summarized in (Figure 1; Table 3).

Chromatographic Conditions for HPTLC

Chromatographic separation was achieved on TLC plates (E. Merck) precoated with silica gel 60 F_{254} (0.2 mm thickness) on aluminum sheet support for both fingerprint development and quantitation studies. Respective samples were spotted using the CAMAG Linomat 5 TLC

Table 1: Results of physicochemical parameters of *M. esculenta* (fruits).

Parameters		Results	Limits
	Foreign organic matter	0.105±0.001	NMT1%
	Loss on drying	14.660±0.060	14.477-14.842
Ash content	Total	3.240±0.047	NMT 5%
	Acid insoluble	2.219±0.028	NMT2.5%
	Water soluble	1.012±0.010	0.982-1.042*
Extractive value	Ethanol soluble	19.652±0.006	NLT15%
	Water soluble	18.475±0.169	NLT17%

Values are (% Mean± S.D., *n*=3); *(prescribed limits)

spotter equipped with a syringe (100 µL; Hamilton). For HPTLC fingerprint development, the sample (10.0 µL) was applied to the plate as a band of 7.0 mm wide and at a distance of 12.0 mm from the edges. Each plate was developed up to a distance of 85.0mm in CAMAG twin trough glass chamber pre-saturated with the mobile phase toluene: chloroform: ethyl acetate: glacial acetic acid (8:2:1.5:0.3 v/v/v/v) for 30 min. After development, the plate was dried in a current of air at room temperature. The plate was derivatized using 10% methanolic sulphuric acid reagent and dried in oven preset at 110°C. All chromatographic separations were performed at 24 ± 1°C. Plates was scanned at 254 nm (deuterium lamp before derivatization), 366 nm (mercury lamp before and after derivatization) and the results were photo documented (Results are depicted in Figure 2). For estimation of gallic acid from samples, the plate was developed in the mobile phase toluene: ethyl acetate: formic acid (4:4:1 v/v/v) respectively. The plates were photodocumented at 254 nm and the spectral analysis for samples were carried out at 272 nm, confirming the presence of gallic acid in samples. The content of gallic acid present in samples was estimated using regression equation obtained from calibration standard curve.

Chromatographic Conditions for HPLC

HPLC analysis was performed using an isocratic HPLC system (JASCO) comprising of binary pumps (PU 1580), rheodyne injector (20 µL loop) and a photodiode array detector (PDAD; MD-1510). Chromatograms were recorded by means of Jasco Borwin chromatography software version 1.50. Separation was achieved on Cosmosil C_{18} column (150 × 4.6 mm, 5.0 µm) using a mobile phase composed of orthophosphoric acid in water: acetonitrile (95:5, v/v., pH-3) delivered at flow rate of 1 mL/min for both estimation of gallic acid and for development of fingerprint. Post equilibration, the samples were injected into HPLC system and the peaks were recorded at 215 nm for gallic acid and maximum absorbance for fingerprint. The developed HPLC method was validated as per ICH guidelines for the estimation of gallic acid from fruits of *M. esculenta*. Figure 3 depicts the HPLC chromatogram of fingerprint for *M. esculenta* fruits.

Method Validation

The developed HPTLC and HPLC methods for estimation of gallic acid were validated using ICH guidelines.[28]

(a) Specificity: Specificity of the method was confirmed by comparing the R_f and retention time (R_t) values of the marker detected in the sample corresponding to the respective reference standards.

(b) Sensitivity: The sensitivity of the method was determined with the signal to noise ratio (S/N) with respect to LOD (S/N of 3:1) and LOQ (S/N of 10:1).

(c) System suitability: System suitability of the developed HPTLC and HPLC method was assessed by developing multiple plates and injections of quality control levels of the reference standards.

(d) Precision and accuracy: Intra- and inter-day precision was evaluated from the triplicate evaluation of quality control samples on the same day and on three consecutive days (n = 3) for both HPLC and HPTLC methods. The acceptable limit for coefficient of variation of values was set at ≤2% for precision and from 85% to 115% for accuracy.

(e) Robustness: The robustness of the method was assessed by deliberately incorporating small variations in the optimized chromatographic conditions in terms of four factors, i.e., change of analyst (analyst 1 and analyst 2), batch of TLC plates / HPLC columns, change in mobile phase composition and change in the

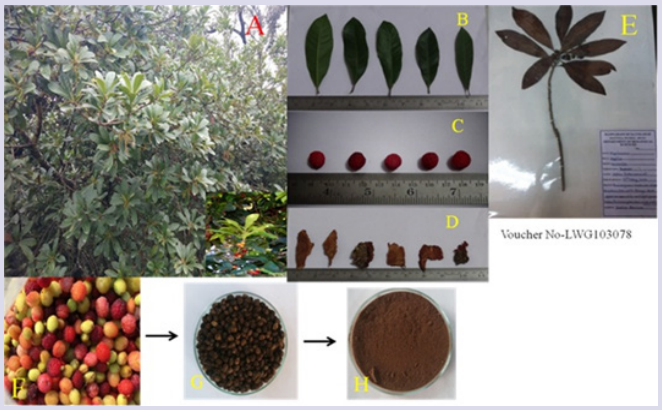

Figure 1: *M. esculenta*: A) habit (inset fruits), B) fresh leaves, C) fresh ripe fruits, D) fresh bark, E) herbaria specimen, F) raw fruits, G) dried fruits, H) dried fruit powder.

Table 2: Results of phytochemicals analysis in *M. esculenta* fruits detected as per preliminary test.

Phytochemical Constituents	Tests	Observation	*Inference
Flavonoids	Ethanolic extract +increasing amount of NaOH	Yellow precipitate was not observed	Absent
	Ethanolic extract + Lead acetate	Yellow precipitate was observed	Present
Tannins	Ethanolic extract + 5% $Fecl_3$	No deep blue colour was observed	Absent
	Ethanolic extract + $K_2Cr_2O_7$	Red precipitate was observed	Present
	Ethanolic extract + Lead acetate	White precipitate was observed	Present
	Ethanolic extract+ $KMnO_4$	Disappearance of pink colour	Present
Alkaloids	Ethanolic extract + Wagner's reagent	Orange brown precipitate was not observed	Absent
Glycosides	Ethanolic extract + 1.0 mL H_2O + NaOH	Yellow coloration	Present
Essential Oils	Ethanolic extract + drops of Vanillin Sulphuric acid	white crystals	Present
Resins	Boiled aqueous extract + conc. H_2SO_4	Reddish brown colour was not observed	Absent

*n=3

flow rate of mobile phase for HPLC. Changes in R_f and R_t were evaluated. A deviation of <5% was set as the acceptance criterion.

(f) **Recovery:** The recovery of the method was evaluated using the standard addition method. Known quantities of reference standards (LQC, MQC and HQC) were added to the sample and extracted using the optimized procedure. Analysis was performed in triplicate and the mean percent recovery was calculated.

Statistical evaluation of the obtained results was carried out using Microsoft Excel 2007. Optimized chromatographic conditions and results for method validation are given in Table 4 and 5 respectively.

ASSAY AND METHOD APPLICATION

The developed method was used to determine the content of Gallic acid from fruits and different morphological parts of *M. esculenta* which were collected from different regions of Uttarakhand, (India). The responses obtained in relation to the R_f in HPTLC and R_t in HPLC were compared with that obtained for the standard Gallic acid. The peak areas from the densitometric scans of HPTLC and the peak areas of the HPLC chromatogram obtained for various samples of *M. esculenta* were correlated to the respective peak areas obtained for the standard Gallic acid. The correlation between the concentration of Gallic acid and the peak area responses is evaluated using regression analysis and the same is applied for estimating the concentration of gallic acid in various samples. Microsoft Excel was used to determine mean, standard deviation, relative standard deviation and mean difference during the analysis.

Estimation of gallic acid from the extracts of different morphological parts of *M. esculenta*

The method was applied to evaluate the content of gallic acid from different morphological parts of *M. esculenta* (leaves, bark, ripe fruits, unripe fruits and seeds) collected from Uttarakhand. The peak of Gallic acid from these samples was identified by comparing their R_f in HPTLC and R_t in HPLC. Results are summarized in (Table 6, Figure 4 and 5)

Estimation of gallic acid from the extract of *M. esculenta*

Table 3: Macroscopical evaluation of *M. esculenta* fruits.

Sr No.	Features	Observation
1	Colour	Fruits- light green (unripe), dark red (ripe) Powder- dark brown
2	Taste	Sweet and sourish
3	Shape	ellipsoid or ovoid, surface tubercled
4	Size	Long- 1.0 cm Diameter- 0.6cm

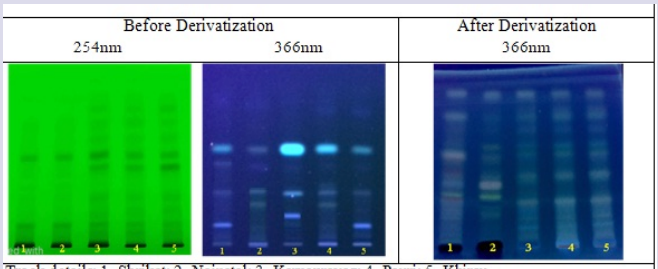

Figure 2: Phytochemical fingerprint of *M. esculenta* (fruits) using HPTLC.

fruit collected from different locations of Uttarakhand

The method was applied to evaluate the content of gallic acid from the fruits of *M. esculenta* collected from different locations of Uttarakhand (Karnaprayag, Khirsu, Shrikot and Pauri, Nainatal). The peak of gallic acid from these samples was identified by comparing their R_f in HPTLC and R_t in HPLC. Results are summarized in (Table 7, Figure 6 and 7).

Estimation of gallic acid from the marketed formulation *Pushyanuga churna* containing *M. esculenta* fruits as one of the ingredient

The method was applied to evaluate the content of Gallic acid from the marketed formulation *Pushyanuga churna* containing *M. esculenta* fruits as one of the ingredient. The peak of Gallic acid from these samples was identified by comparing their R_f in HPTLC and R_t in HPLC. Results are summarized in (Table 8, Figure 8 and 9).

RESULTS AND DISCUSSION

M. esculenta fruits have been evaluated for their phytochemical constituents to develop parameters that would reflect their quality. Proximate analysis of fruits of *M. esculenta* for parameters such as foreign organic matter, loss on drying, ash values (total, acid insoluble and water soluble) and extractive values (ethanol soluble and water soluble) indicate that the values are within the permissible limits. The ethanol soluble extractables were found to be relatively higher indicating the presence of polar components in the *M. esculenta* fruits (Table 1). Chemical tests to qualitatively evaluate the profile of secondary metabolites indicate the presence of flavonoids, tannins and phenolics, essential oil, glycosides, terpenoids in the fruits (Table 2). Typical morphological characters of *M. esculenta* fruits are listed in Table 3 and is depicted in Figure 1.

Phytochemical fingerprints for the fruits of *M. esculenta* have been developed using both HPTLC and HPLC techniques. Gallic acid has been estimated from ethanolic extracts of *M. esculenta* fruits using both the chromatographic techniques. The R_f of Gallic acid is found to be 0.37 in HPTLC while the R_t for gallic acid is found to be 4.3 min in HPLC. HPTLC method was validated for the concentration range of 10 – 500 µg/mL with an LOQ of 10 µg/mL whereas the HPLC method was validated for a linearity range of 0.5 – 50 µg/mL with a LOQ of 0.5 µg/mL. Prashar and Patel, 2020, have reported an HPTLC technique to quantify gallic from the fruits of *M. esculenta*, but the mobile phase developed in our study is simpler, with better band resolution compared to the reported work (Figure 6 and 7). The LOQ reported in our study is comparatively

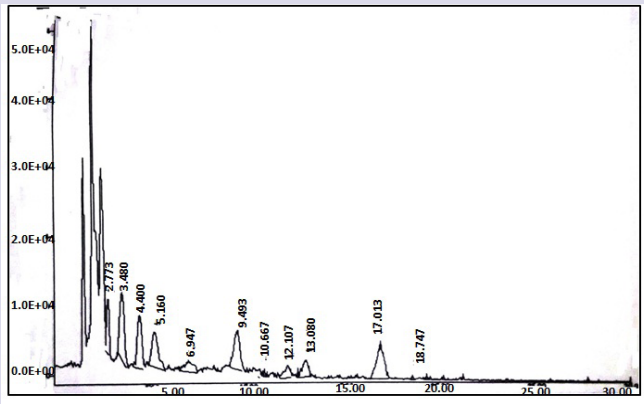

Figure 3: Phytochemical fingerprint of *M. esculenta* (fruits) using HPLC.

Table 4: Optimized chromatographic conditions for estimation of gallic acid using HPTLC and HPLC techniques.

Parameters	HPTLC	HPLC
Stationary phase	HPTLC plates precoated with silica gel 60 F_{254}	Cosmosil C_{18}
Instrument	CAMAG LinomatV Automatic Sample Spotter	Jasco
Mode of separation	Normal phase	Reversed phase
Mobile phase	Toluene: chloroform: ethyl acetate: glacial acetic acid (fingerprint) Toluene:ethylacetate:formic acid (quantitation)	Water (0.5% orthophosphoric acid): acetonitrile (fingerprint and quantitation)
Development chamber	CAMAG twin trough	NA
Chamber saturation (min)	30	NA
Band length (mm)	7	NA
Flow rate(mL/min)	NA	1.0
Pump, injector	NA	PU-1580, Rheodyne
Spotting/ injection volume (µL)	10.0	20.0
Scanner/ detector	CAMAG TLC Scanner IV	PDA, MD-1510
Lamp	Deuterium and mercury	NA
Derivatization, photo documentation	10%methanolic sulphuric acid reagent (for fingerprint analysis), CAMAG Reprostar 3	NA
Wavelength (nm)	254, 272, 366	215, maximum absorbance
Software	Win CATS planar chromatography manager software v. 1.4.7	Jasco-Borwin Chromatography software, v. 1.5

Note: NA-Not applicable

Table 5: Results of method validation experiment for estimation of gallic acid using HPTLC and HPLC.

Parameters	HPTLC	HPLC
Mobile phase	Toluene:ethylacetate: formic acid 4:4:1v/v/v	Orthophosphoric acid in water: acetonitrile (95:5 v/v; pH-3.0)
R_f/ R_t	0.37	4.3
LOD and LOQ (µg/mL)	8 and 10	0.1 and 0.50
Linearity (µg/mL)	10-500	0.50-50
Regression equation	y = 127.9x + 60.87	y= 115408x-29112
Coefficient of determination (r^2)	0.999	0.999
Intraday Precision (% CV) LQC, MQC, HQC	0.97, 0.49, 0.21	0.97. 0.38. 0.78
Interday Precision (% CV) LQC, MQC, HQC	1.26, 0.73, 0.96	0.78, 0.61, 0.99
Recovery (%)	92.49-95.26	94.72-96.45
Specificity Ruggedness	Specific, rugged	Specific, rugged

*Mean±SD, *n*=3

less, indicating that the method validated in this study is more sensitive than that in the previous paper.

The content of gallic acid has been estimated from different morphological parts of *M. esculenta* (Table 6, Figure 4 and 5) and was found to be maximum in unripe fruits of *M. esculenta* (167.15µg/10mg by HPTLC and 182.06 µg/10mg by HPLC). Earlier studies[22] have reported gallic acid content of 129.3 µg/10mg of methanolic extract which is less than our estimates. This could be attributed to the use of ethanolic extract of fresh powders of *M. esculenta* fruits, the collection of which had been started since 2016 with yearly collections of fruits from various regions of Uttarakhand, India.[20] Our repeated studies have been performed with fresh fruit samples collected in every subsequent year, which could be one of the reasons for higher phytochemical contents. These results are positively correlated with our unpublished observation[29] which was used as baseline data to design efficacy study of PCOS using fruits of *M. esculenta*.[30] Stability studies on the plant raw material and extracts are in process.

The content of gallic acid estimated by HPTLC and HPLC are comparable. The HPLC estimation shows similar values of gallic acid content as that is estimated by HPTLC, which confirms the Gallic acid content in various samples. Gallic acid content, when estimated from *M. esculenta* fruit samples collected from different regions of Uttarakhand,

shows distinctive regional variations with the fruits collected from Shrikot showing maximum content (Table 7, Figure 6 and 7). This could be attributed to the variation in climatic conditions of the forests from where fruits have been collected. The method has also been applied to estimate the content of gallic acid from a marketed traditional Ayurvedic formulation *Pushyanuga churna*, as it contains *M. esculenta* fruits as one of the ingredients. The content of gallic acid was found to be 0.640 mg/g with HPTLC and 0.742 mg/g with HPLC (Table 8, Figure 8 and 9). Since gallic acid is a ubiquitous phytochemical, its source in the formulation could be from many of the ingredient plant raw materials including *M. esculenta* fruits. Considering the label claim of the manufacturer, the formulation should contain at least 0.07 mg of gallic acid, corresponding to the content estimated in the *M. esculenta* fruits alone. Since, the estimated gallic acid content in the formulation is higher; we can assume the addition of adequate amount of *M. esculenta* fruits in the formulation. For complete quality evaluation of the formulation however, the Gallic content of each ingredient plant raw material will have to be ascertained before we could apply the total Gallic acid content as a reliable quality

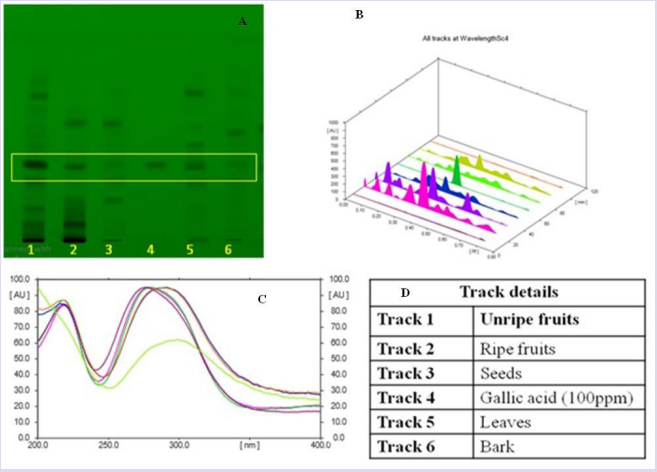

Figure 4: A: Plate photo for estimation of gallic acid from different morphological parts of *M. esculenta* at 254nm; B: Densitometric scan of different morphological parts of *M. esculenta* at 272nm; C: Spectral scan for confirming the presence of gallic acid from different morphological parts of *M. esculenta*; D: Track details of sample spotted on HPTLC plate.

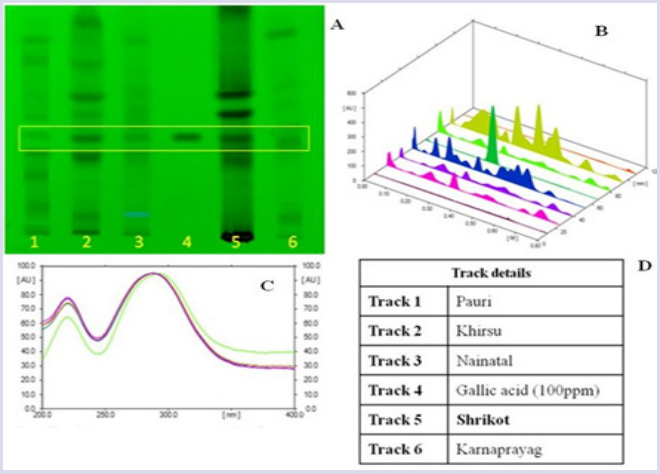

Figure 6: A: Plate photo for estimation of gallic acid from *M. esculenta* fruits collected from different locations of Uttarakhand at 254nm; B: Densitometric scan of *M. esculenta* fruits collected from different locations of Uttarakhand at 272nm; C: Spectral scan for confirming the presence of gallic acid from *M. esculenta* fruits collected from different locations of Uttarakhand; D: Track details of sample spotted on HPTLC plate.

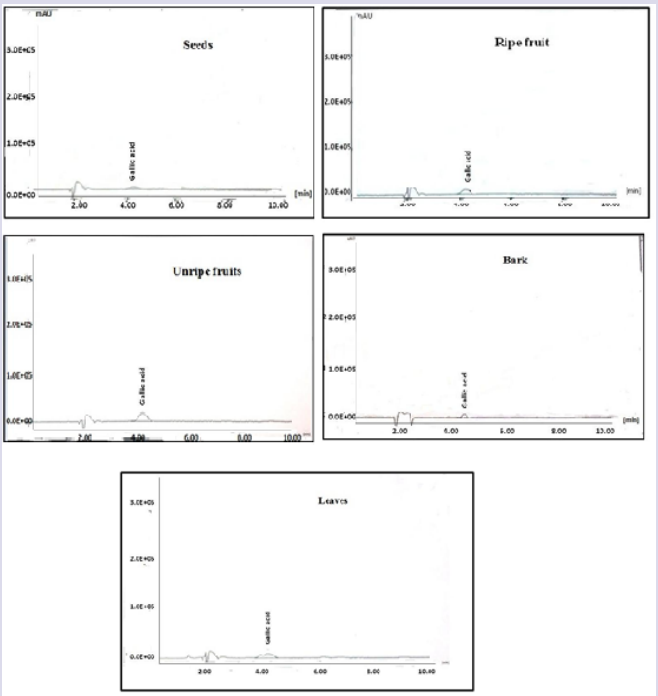

Figure 5: Estimation of gallic acid from different morphological parts of *M. esculenta* at 215 nm using HPLC.

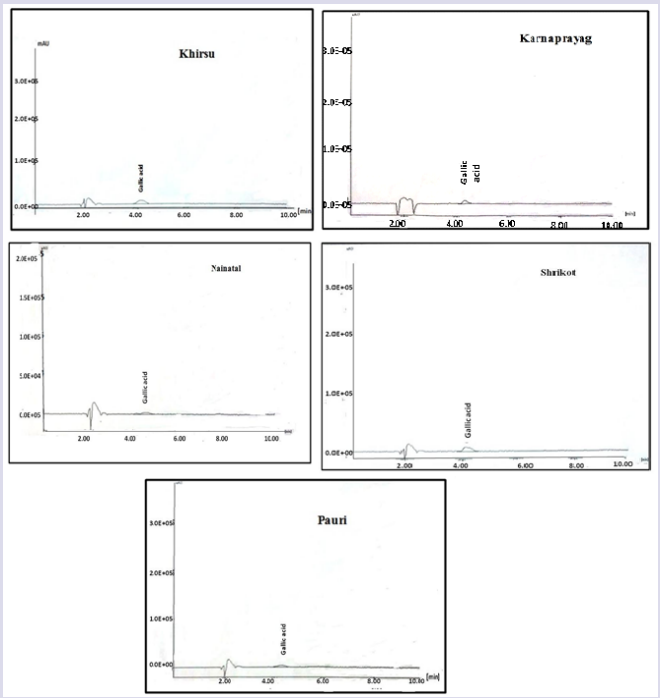

Figure 7: Estimation of gallic acid from *M. esculenta* collected from different locations of Uttarakhand at 215nm using HPLC.

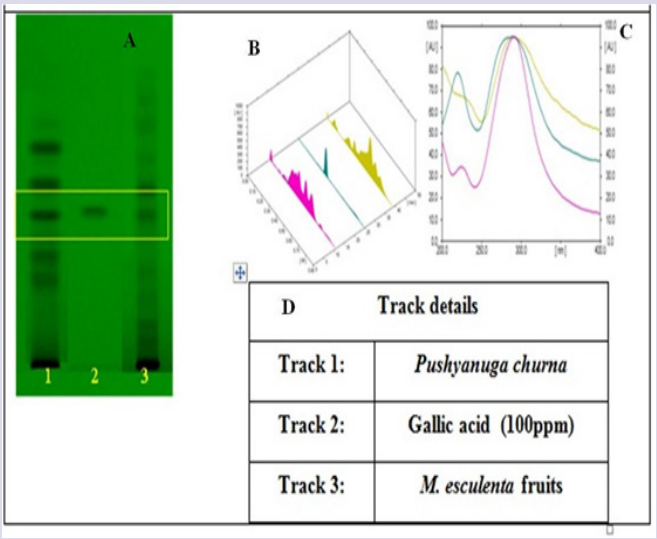

Figure 8: A: Plate photo for estimation of gallic acid from marketed formulation *Pushyanuga churna* containing *M. esculenta* fruits as one of the ingredient at 254nm; B: Densitometric scan of marketed formulation and *M. esculenta* at 272nm; C: Spectral scan for confirming the presence of gallic acid from marketed formulation and *M. esculenta*; D: Track details of sample spotted on HPTLC plate.

Table 6: Results for estimation of gallic acid from the *M. esculenta* from different morphological parts.

Different morphological parts	HPTLC (µg/10mg)*	HPLC (µg/10mg)*
Leaves	102.04±0.01	119.96±0.05
Bark	108.07±0.01	123.15±0.09
Seeds	96.88±0.01	104.03±0.01
Ripe fruit	143.01±0.01	150.10±0.01
Unripe fruit	167.16±0.01	182.07±0.02

*Mean±SD, *n*=3

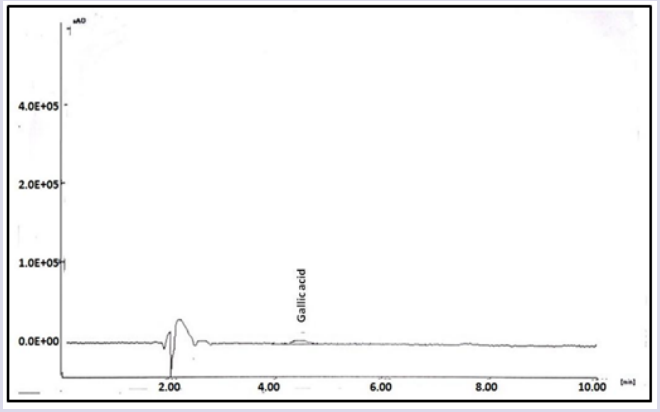

Figure 9: Estimation of gallic acid from marketed formulation *Pushyanuga churna* containing *M. esculenta* fruits as one of the ingredient at 215 nm using HPLC.

Table 7: Results for estimation of gallic acid from *M. esculenta* fruits collected from different locations of Uttarakhand, India.

Sample from different locations of Uttarakhand, India	HPTLC (µg/10mg)*	HPLC (µg/10mg)*
Karnaprayag	137.11±0.04	144.46±0.05
Khirsu	156.30±0.01	153.71±0.02
Shrikot	159.34±0.02	166.15±0.05
Pauri	126.95±0.16	129.02±0.02
Nainatal	114.87±0.01	128.35±0.03

*Mean±SD, *n*=3

Table 8: Result for estimation of gallic acid from *Pushyanuga churna*, marketed formulation.

Samples	HPTLC (mg/g)*	HPLC (mg/g)*
Pushyanuga churna	0.64±0.01	0.74±0.01

*Mean±SD, *n*=3

control parameter for *Pushyanuga churna*. Such development of quality control parameters based on the content of phytochemical markers is needed to enable the traditional formulations to receive global acceptance.

CONCLUSION

The methods of HPTLC and HPLC developed and validated in the current study are simple, precise, accurate and sensitive and can be used as quality-control tools for plant extracts or poly-herbal combination containing *M. esculenta* fruits. This will greatly aid in standardization and prevention of its adulteration. Although *M. esculenta* is distributed widely along the mid-Himalayan regions, the commercial use of its fruits as a medicinal raw material has been limited especially due its seasonal availability and short shelf life. The current study highlights the potential of the fruit to be developed into a value-added minor forest produce for the Garhwal Himalayas, by initiating collection, storage, drying and powdering of fruits by local communities. The phytochemical value of unripe fruits as demonstrated in the current study supports the collection of unripe fruits which will enable extending the shelf life of the collected fruits till further processing. Organized harvest of *M. esculenta* will also ensures sustainable use of an important medicinal tree which has a significant value in biodiversity of the Garhwal Himalayan region. The data generated in the current study will be a valuable addition to the current knowledge about phytochemistry of *M. esculenta* and the validated chromatographic techniques can be applied effectively as quality control tools to ensure the use of authentic sample of *M. esculenta* in many herbal formulations where it is a key ingredient.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HPTLC: High Performance Thin Layer Chromatography; **HPLC:** High Performance Liquid Chromatography; **LOD:** Limit of detection; **LOQ:** Limit of Quantification; **LQC:** Low Quality Control Concentration; **MQC:** Mid Quality Control Concentration; **HQC:** High Quality Control Concentration; **NLT:** Not Less Than; **NMT:** Not More Than; **SD:** Standard Deviation; **v/v:** volume by volume; $\mathbf{R_t}$**:** Retention time; $\mathbf{R_f}$**:** Retardation factor.

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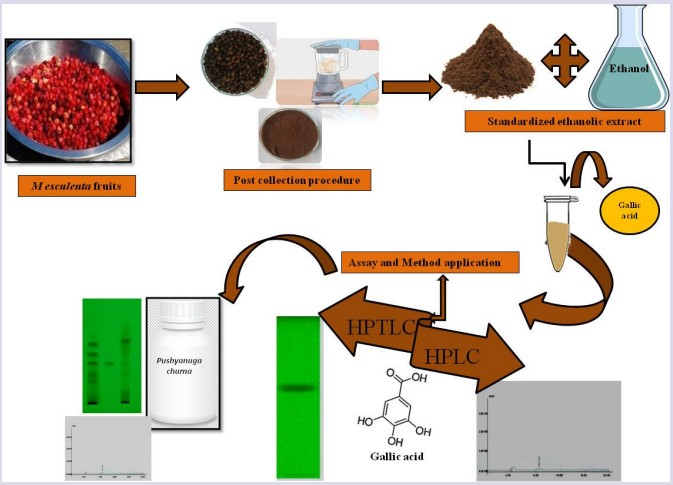
PICTORIAL ABSTRACT



SUMMARY

- *Myrica esculenta* fruits and its different morphological parts were collected from forests of Uttarakhand (India).
- Standardized ethanolic extract was prepared and pharmacognostic parameters were evaluated.
- HPTLC and HPLC methods were developed and validated as per ICH guidelines to quantify Gallic acid.
- The bioactive marker (Gallic acid) content was estimated from standardized ethanolic extracts of fruits collected from different regions of Uttarakhand (India) and different morphological parts using HPTLC and HPLC.
- Gallic acid was also estimated from a traditional Ayurvedic formulation (Pushyanuga churna) as it contains *M. esculenta* using HPTLC and HPLC.
- Both HPTLC and HPLC methods of Gallic acid estimation provide sensitive quality evaluation methods.

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