

LC-MS/MS-based Targeted Metabolomic Profiling of Aqueous and Hydro-alcoholic Extracts of *Pistacia integerrima* Linn., *Quercus infectoria* Olivier and *Terminalia chebula* Retz

Pharmacognosy Magazine

19(2) 222–230, 2023

© The Author(s) 2023

Article reuse guidelines:

in.sagepub.com/journals-permissions-india

DOI: 10.1177/09731296221144809

journals.sagepub.com/home/phm

Anil Kumar Saini¹, Laxman Sawant¹, Sultan Zahiruddin², Dhiraj Shrivastva¹,
Ranjan Mitra¹, Rajiva Kumar Rai¹ and Sayeed Ahmad²

Abstract

Background: *Pistacia integerrima* (PI) galls, being rich in polyphenolic compounds, owing to its high restorative worth and restricted assets and exorbitant utilization lead to the overexploitation of the species and availability issues. It is expected to categorise under the red list of threatened taxa. Ayurveda text *Bhaishajya Ratnavali* mentioned *Quercus infectoria* (QI) as 'pratinidhi' or substitute of PI and *Yogaratanakara* (abhavvarga) text reference indicated *Terminalia chebula* (TC) having the same therapeutic character as in PI. Hence, this study was focused on the comparative quantification of major polyphenolic compounds in aqueous and hydro-alcoholic extracts of PI, QI and TC by liquid chromatography and tandem mass spectroscopy (LC-MS/MS).

Objectives: The LC-MS/MS method was used for the determination of polyphenolic compounds in aqueous and hydro-alcoholic extracts of PI, QI and TC.

Materials and Methods: The LC-MS/MS method was developed for the quantification of targeted polyphenolic compounds and validated in terms of linearity, accuracy, precision, limit of detection and limit of quantification. Analysis performed by LC-MS/MS 6460 C triple quad coupled with Agilent HPLC. The chromatographic separation was performed on the RP C₁₈ column with gradient elution.

Results: The developed method of LC-MS/MS was used for the quantitative estimation of gallic acid, corilagin acid, caffeic acid, syringic acid, rutin hydrate, ellagic acid, quercetin and morin hydrate in PI, QI and TC. As per method validation parameters, calibration plots were found to be linear, accurate, precise and specific for LC-MS/MS. The calibration plot exhibited an excellent coefficient of determination ($R^2 \geq 0.99$), and extraction recovery was found 96.20%–103.71%. Gallic acid and ellagic acid are the most abundant compound among eight targeted polyphenolics in all extracts.

Conclusion: The finding of LC-MS/MS analysis of polyphenolic compounds in aqueous and hydro-alcoholic extracts indicates that PI, QI and TC are having phytochemical similarities up to an extent in polyphenolic compounds as a secondary metabolite; however, a firmer opinion can be formed after a comparative efficacy study and the evaluation of all three extracts.

Keywords

Polyphenolic compounds, LC-MS/MS, *Pistacia integerrima*, *Quercus infectoria*, *Terminalia chebula*.

Introduction

Pistacia integerrima Linn. (PI) which belong to the Anacardiaceae family are deciduous aromatic shrubs and trees, slightly bitter and astringent with terebinthine in odour.¹ It is native to Asia and has a vast range of distribution from East Afghanistan to Pakistan and the Himalayas in the Northwest and West, as well as Kumaon.² The majority of phytochemicals in PI are polyphenolic compounds.¹ Along with essential oil, alkaloids, saponins and sterols, these are

thought to be responsible for the majority of the plant's therapeutic activities.³

¹Dabur Research and Development Centre, Dabur India Limited, Sahibabad, Ghaziabad, Uttar Pradesh, India

²Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India

Corresponding author:

Sayeed Ahmad, Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India.

E-mail: sahmad_jh@yahoo.co.in



PI galls were traditionally used to treat stomach and dental problems, sickle cell anaemia, skin issues (psoriasis, vitiligo, etc.), asthma and fever. Polyphenolic compounds have been demonstrated to exhibit a wide range of biological and pharmacological activity, including insecticidal, anthelmintic, anti-plasmodial, anti-sickling, cytotoxic, anti-inflammatory, and antibacterial activity, hepatoprotective, and anticonvulsant properties.^{4–12} PI galls are extensively used as a herbal medicinal in the formulation of Ayurvedic and Chinese medicines and are said to provide therapeutic activity for a variety of respiratory ailments. High medicinal/therapeutic value and commercial demand of PI galls in the national and international market leads to overexploitation of the species. In some countries, PI has been categorised under the list of threatened, critically endangered species and its vulnerable conservation status due to overexploitation and habitat degradation.^{13,14}

Quercus infectoria Oliv. (QI) galls (family- Fagaceae) are the spherical shaped gallnuts formed by the gall wasp, *Cynips gallae-tinctoriae* Oliv in small shrub or trees.¹⁵ Galls are recommended as representative of PI in the Ayurveda textbook *Bhaishajaya Ratnavali* (Abhavparkaran). Traditionally, QI galls are used as a substitute in formulations (*Karkatadichurna*, *Balachaturbhadra*, *Kantkaryavaleha*, *Shringyadichurn*).^{16,17} These are recommended for the treatment of allergic and infectious diseases such as asthma, dysentery, skin infections and anti-inflammatory diseases.¹⁸ The screening of QI galls predominantly shows that it is rich in polyphenolic compounds such as tannins (50%–70%), gallic acid, ellagic acid and flavonoids. Alkaloids, carbohydrates, glycoside, saponins, sterols, triterpenes are also reported as part of phytoconstituents.^{19,20} Polyphenolic compounds in QI galls pharmacologically showed significant activity as anti-inflammatory, astringent, anti-diabetic, anti-tremorine, anti-viral, anti-fungal, antioxidant, nephroprotective and chemopreventive activities.^{15,21–32}

Terminalia chebula Retz. (TC) belonging to the family Combretaceae is a medium-to-large size deciduous tree; it is regarded as ‘King of Medicine’ by ayurvedic apothecaries and traditional medicinal practitioners. TC significantly contains 32%–34% of tannins, gallic acid, ellagic acid, chebulinic acid, flavonol glycosides, triterpenoids and other phenolic compounds such as ethyl gallate, luteolin, protein, amino acids and minerals.³³ Similar to QI and PI, TC fruits are also rich in high phenolic components. They are actively used in various classical and proprietary formulations. They have been traditionally used for different therapeutic benefits and proved pharmacologically to be active as antioxidant, anti-bacterial, anti-fungal, anti-viral, anti-protozoal, anti-carcinogenic, radioprotective, anti-mutagenic, anti-arthritis, chemopreventive, hepatoprotective, cardioprotective, cytoprotective, anti-ulcer, anti-diabetic, anti-inflammatory, hypolipidemic, antispasmodic, wound healing, purgative, anti-allergic, immunomodulation, bronchial asthma and atopic dermatitis.^{34–36}

In the Ayurveda textbook, *Yogratnakar* (The First Schedule) in ‘Abhavvarga’ mentions ‘PI’ as having the same therapeutic character as that of TC.³⁷ Polyphenolic compounds of various plants and species are therapeutically proven to be active against various diseases. PI, QI and TC are rich in various polyphenolic compounds. However, there have been no previous published data on the ‘comparative study of polyphenolic compounds’ as a secondary metabolite. A vast amount of literature are available for the identification and quantification of polyphenolics in *P. integerrima*, *Q. infectoria* and *T. chebula*. Hence, the present study has been designed for the comparative analysis of polyphenolic compounds in aqueous and hydro-alcoholic extracts of QI galls and TC fruits with PI galls by using the liquid chromatography and tandem mass spectroscopy (LC-MS/MS) technique.

The study involves the identification of targeted polyphenolic compounds in the aqueous and hydro-alcoholic extracts of the PI galls, QI galls and TC fruits using LC-MS/MS. Targeted polyphenolic compounds were identified through multiple reaction mode (multi-MRM) scanning by using the MS/MS analysis technique. Hence, the study provides a reference data of common compounds as ‘polyphenolic compounds’ in QI galls and TC fruit with respect to PI galls.

Materials and Methods

Gallic acid (99%), corilagin (99%), caffeic acid (99.5%), syringic acid (95%), rutin hydrate (95%), ellagic acid (99.8%), quercetin (97%) and morin hydrate (90%) were purchased from Sigma Aldrich, India. HPLC grade Methanol, LC-MS grade acetonitrile and formic acid were procured from Merck Life Science Pvt. Ltd., Mumbai, India. Ultrapure water was obtained by using a Milli-Q Advantage® A10 and Millipore Elix® (Elix Technology) LC Pack water purification system.

Plant Materials

Dried galls of PI, QI and dried fruit pericarp of TC were arranged from the approved inventory of Dabur India Ltd., Sahibabad (Ghaziabad), India, and authenticated by Dr C. S. Rana (Taxonomist) of the Bio Resource Development Department, against the Voucher Nos. DRDC-1256-BRD/P. *Integerrima* L., DRDC-1257-BRD/QI and DRDC-1255-BRD/TC.

Preparation of Aqueous Extracts

Dried galls of PI, QI and fruit pericarp of TC were pulverised to get coarse ground powder. For the aqueous extraction of each drug, the decoction method (classical method) was adopted. Demineralised water (DM) was added in the coarse ground powder in a stainless-steel extractor and boiled for 2

hours and filtered off through (60 mesh) 149 μm nylon cloth. The process was repeated twice for complete extraction. The obtained filtrate was concentrated in a vacuum tray drier at 45°C under reduced pressure. After drying, lumps and flakes of dry extract were grounded under controlled conditions of temperature (25°C $\pm$ 2°C) and relative humidity (45% $\pm$ 5% RH) to obtain a fine powder. The same process was implemented for all three drugs to obtain an aqueous extract.

Preparation of Hydroalcoholic Extracts

Dried galls of PI, QI and fruit pericarp of TC were pulverised to obtain the coarse ground powder. The coarse ground powder was taken in a round bottom flask fitted with a condenser and refluxed for 5 hours using water alcohol (50:50) as a solvent ratio. Then it was filtered through (60 mesh) 149 μm nylon cloth to get the filtrate. The process was repeated twice after 3 hours by using a fresh solvent till the drug appeared exhausted. The obtained filtrate volume was concentrated by a vacuum tray drier at 45°C and under reduced pressure. After drying, the extract was scrapped from the tray in the form of hard lumpy mass and flakes. Lumps/flakes of the extract were crushed under controlled conditions 25°C $\pm$ 2°C/45% $\pm$ 5% RH into a fine powder. All aqueous and hydroalcoholic extracts were stored in airtight containers for further use. The same process was implemented for all three drugs to obtain the hydro-alcoholic extract.

Preparation of Standard Solutions

Accurately about 2 mg of each reference standard (caffeic acid, corilagin, ellagic acid gallic acid, morin hydrate, quercetin, rutin hydrate and syringic acid) of known purity was weighed in 15 mL of methanol and sonicated for 5 min to dissolve the content completely. The volume was made up with methanol up to 25 mL. Before analysis, all the stock solution was mixed to obtain a concentration of 200 $\mu\text{g/mL}$ for each marker and filter using a 0.2 μm polytetrafluoroethylene (PTFE) membrane filter.

Preparation of Sample Solutions

An accurately weighed dry extract of 500 mg in the powder form of the plant material was taken in a 25 mL volumetric flask, with a little amount of methanol, and sonicated for 10 min at 25°C. After solubilisation, the volume was made up to 25 mL with methanol to obtain a concentration of 20 mg/mL. The extract solution was filtered through the AXIVA-ETO-sterile PTFE membrane filter (0.45 μm) and filled in sample vials prior to injection. These vials were injected in triplicate into the HPLC-MS/MS system to determine the content of compounds.

LC-MS/MS Instrumentation and Chromatographic Conditions

Agilent Technologies was used to conduct the LC-MS/MS-6460 analysis of the polyphenolic compounds with a triple quad coupled with the Alliance HPLC system (e2695 Separation module, Waters, United States) equipped autosampler attached with a PDA detector (Waters 2998). The separation was carried out on an Agilent Eclipse plus reversed phase C₁₈ column (3.5 μm , 4.6 $\times$ 100 mm²). The temperature of the column was set to 40°C. The elution gradient contains mobile phases A (0.1% v/v formic acid in acetonitrile) and B (0.1% v/v formic acid in water). The flow rate was set to be 0.6 mL/min, and the injection volume was 4 μL for gallic acid and 10 μL for other compounds and samples. The elution gradient was carried out in the following programme using a mixture of mobile phases A and B: 0–7 min, 98% B; 7–9 min, 80% B; 9–15 min, 50% B; 15–20 min, 30% B; 20–21 min, 5% B; 21–25 min, 5% B; 25–26 min, 98% B; 26–35 min, 98% B.

The Agilent LC-MS/MS 6640 model triple quadrupole mass spectrometer equipped with a jet stream electrospray (AJS-EIS) was used for MS detection in both positive and negative ionisation modes. To quantify the analytes, multiple reaction mode (MRM) was used: the assay of studied substances was done after two or three transitions, the first for quantitative reasons and the second and/or third for confirmation. For AJS-ESI-QQQ, the set parameters are as follows. Nitrogen gas was used as collision gas. The capillary voltage was 3.5 kV in positive and negative modes, the nozzle voltage was 0.6 kV, the ion source/gas temperature was set to 325°C, the gas flow was 10 l/min, the nebuliser pressure was 35 psi, the drying gas temperature was 350°C and the sheath gas flow was 11 l/min.³⁸

Validation of the LC-MS/MS Method

Linearity, the Limit of Detection and the Limit of Quantification (LOQ)

A calibration study was conducted over a wide concentration range of 0.1–0.2 $\mu\text{g/mL}$ for 1–8 compounds (by using a stock solution of mix standards). Limit of detections (LODs) and limit of quantifications (LOQs) for each standard substance were acquired, while the signal-to-noise ratios were 3 and 10, respectively.^{39,40}

Precision and Recovery

The precision was analysed using standard solutions with six replicates, and each standard compound's peak area's percentage relative standard deviation (%RSD) was calculated. Standard addition to a specified extract was used to test the method's accuracy.^{39,40} The recovery was calculated

using the following equation: recovery (%) = (amount found – original amount)/amount spiked $\times$ 100%.

Robustness

The robustness of the new LC-MS/MS technique was evaluated by changing the mobile phase flow rate and column temperature. The method's robustness was determined by calculating the %RSD of the peak region.^{39,40}

Determination of Selected Polyphenolic Compounds in Extracts

The developed LC-MS/MS method was applied to analyse gallic acid, corilagin acid, caffeic acid, syringic acid, rutin hydrate, ellagic acid, quercetin and morin hydrate content in the aqueous and a hydro-alcoholic extract of PI, QI and TC. The corresponding concentration was determined using the standard curve's regression equation.

Results and Discussion

Optimisation of LC-MS/MS Conditions

The LC-MS/MS analysis conditions were used for the simultaneous and comparative quantification of the eight targeted polyphenolic markers found in aqueous and hydro-alcoholic extracts of PI, QI and TC. The comparative analysis was performed with a reversed-phase C₁₈ column (3.5 μ m, 4.6 $\times$ 100 mm²) at 40°C of column temperature. Elution gradient consisted of mobile-phase systems A and B as 0.1% v/v formic acid in acetonitrile and 0.1% v/v formic acid in water, respectively, at a flow rate of 0.6 mL/min with 10 μ L injection volume of each sample. Compounds 1–8 were detected in 35 min using the LC-MS/MS technique (Table 1, Figure 1).

Figure 1 shows the comparative analysis of LC-MS/MS for polyphenolic compounds. The ESI source was used in the

Table 1. Detection Parameters of Polyphenolics Compound by LC-MS/MS Method.

Sl. No.	Compound	Rt (min.)	Molecular Formula	Mol. Wt.	Precursor Ion (m/z)	Quantifier (m/z)	Qualifier (m/z)	Ion mode
1	Gallic acid	4.905	C ₇ H ₆ O ₅	170.12	168.8	124.8	106.7, 79	–ve
2	Corilagin acid	12.61	C ₂₇ H ₂₂ O ₁₈	636.46	632.3	300.7	292.9, 235.6	–ve
3	Caffeic acid	12.932	C ₉ H ₈ O ₄	180.16	181	162.9	89.2	+ve
4	Syringic acid	13.173	C ₉ H ₁₀ O ₅	198.17	197	182	123	–ve
5	Rutin hydrate	13.397	C ₂₇ H ₃₀ O ₁₆	610.51	611.1	464.9	302.9	+ve
6	Ellagic acid	13.715	C ₁₄ H ₆ O ₈	302.19	300.9	257	229, 185	–ve
7	Quercetin	16.038	C ₁₅ H ₁₀ O ₇	302.24	301	178.7	151	–ve
8	Morin hydrate	16.039	C ₁₅ H ₁₀ O ₇	302.23	300.7	292.9	172.2, 150.6	–ve

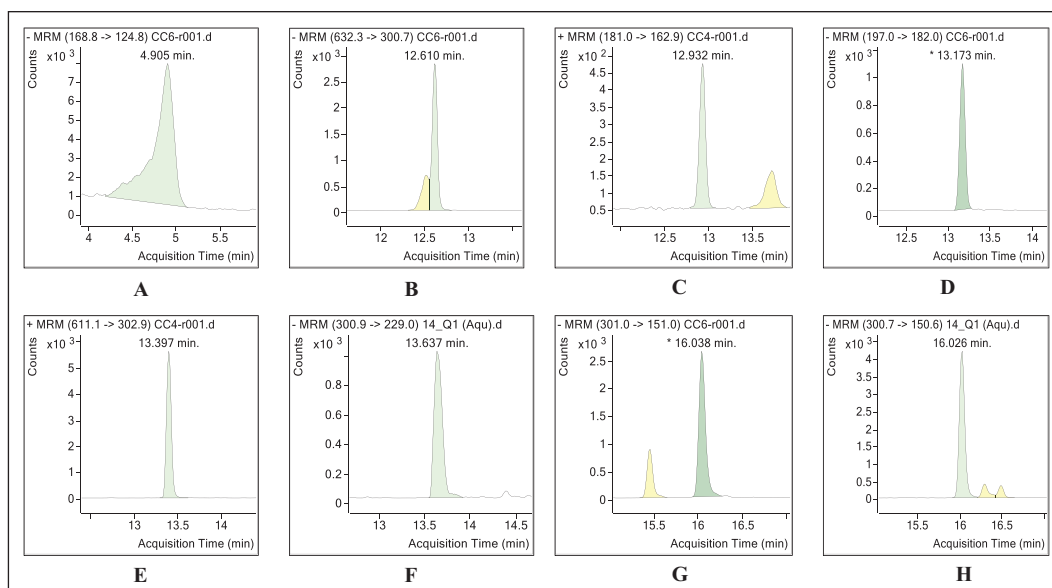


Figure 1. LC-MS/MS Representative Chromatograms of the Polyphenolics Compound.

MRM (negative and positive ion modes). Precursor ions for compounds 1 and 7 were detected at m/z 168.8 and 301 in the positive ion mode $[M + H]^+$, respectively, whereas compounds 2, 3, 4, 5, 6 and 8 were detected at m/z 632.3, 181, 197, 611.1, 300.9 and 300.7 in the negative ion mode $[M - H]^-$, respectively (Figure 2). MRM conditions optimised for LC-MS/MS are provided in Table 1.

Validation of LC-MS/MS Method

Calibration Curve

The calibration graph's linear regression data revealed a good linear relationship between the peak area and a wide range of

selected concentrations. The linear regression calibration curves were plotted between the peak area against the concentration and were linear from 100 to 2,000 ng/mL for all selected targeted markers, namely gallic acid, corilagin acid, caffeic acid, syringic acid, rutin hydrate, ellagic acid, quercetin and morin hydrate with good linear relationships of 0.998 ± 0.001 , 0.992 ± 0.004 , 0.997 ± 0.001 , 0.991 ± 0.004 , 0.996 ± 0.003 , 0.993 ± 0.003 , 0.998 ± 0.001 , and 0.992 ± 0.002 , respectively (Table 2).

Precision

Precisions were calculated and reported in %RSD. Precision data comprise inter-day, intra-day and inter-analyser

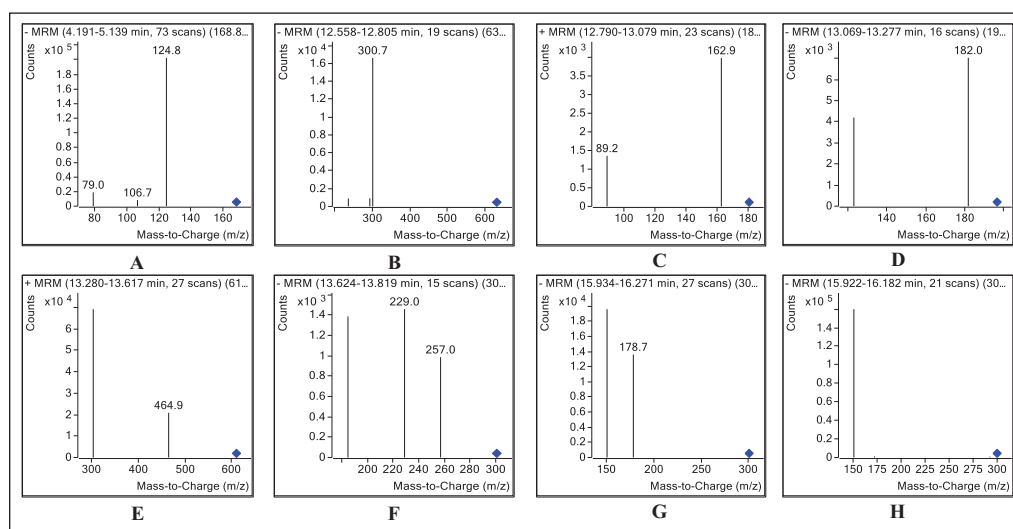


Figure 2. Mass Spectra Showing the Transitions from Precursor to Daughter Ions for Polyphenolics Compound.

Table 2. Calibration Curves, LOD and LOQ of Polyphenolics Compound by LC-MS/MS Method.

Sl. No.	Compound	Calibration Curves	Regression $\pm$ S.D.	Slope $\pm$ S.D.	Intercept $\pm$ S.D.	Linearity Range (μ g/mL)	LOD (μ g/mL)	LOQ (μ g/mL)
1.	Gallic acid	$Y = 61074.98x + 2959.57$	0.998 ± 0.001	61074.98 ± 0.402	2959.57 ± 0.255	0.1–2.0	0.032	0.115
2.	Corilagin acid	$Y = 6650.92x - 1403.37$	0.992 ± 0.004	6650.92 ± 0.075	1403.37 ± 0.021	0.1–2.0	0.024	0.073
3.	Caffeic acid	$Y = 2411.70x + 118.38$	0.997 ± 0.001	2411.70 ± 0.102	118.38 ± 0.006	0.1–2.0	0.022	0.065
4.	Syringic acid	$Y = 2373.10x + 82.52$	0.991 ± 0.004	2373.10 ± 0.082	82.52 ± 0.008	0.1–2.0	0.023	0.069
5.	Rutin hydrate	$Y = 26016.0x + 1180.19$	0.996 ± 0.003	26016.09 ± 0.610	1180.19 ± 0.085	0.1–2.0	0.027	0.082
6.	Ellagic acid	$Y = 545.43x + 135.67$	0.993 ± 0.003	545.43 ± 0.024	135.67 ± 0.004	0.1–2.0	0.030	0.092
7.	Quercetin	$Y = 6262.27x + 1293.26$	0.998 ± 0.001	6262.27 ± 0.135	1293.26 ± 0.046	0.1–2.0	0.024	0.072
8.	Morin hydrate	$Y = 49530.1x + 18779.05$	0.992 ± 0.002	49530.14 ± 0.486	18779.05 ± 0.072	0.1–2.0	0.031	0.093

measurements. The %RSD less than 2 showed that the precision measurement yields satisfactory results and confirmed that the method can be used in any laboratory for the estimation of caffeic acid, corilagin, ellagic acid, gallic acid, morin hydrate, quercetin, rutin hydrate and syringic acid (Table 3). The precision acceptance criteria were based on ICH guidelines and industry-accepted practices.⁴¹

Accuracy

The method has proved that the recoveries of the gallic acid, corilagin acid, caffeic acid, syringic acid, rutin hydrate, ellagic acid, quercetin and morin hydrate in samples were within the range of 96.20 ± 1.16 , 102.20 ± 1.43 , 99.20 ± 0.91 , 103.50 ± 0.55 , 99.50 ± 1.45 , 103.71 ± 0.64 , 99.10 ± 1.22 , and 99.50 ± 1.19 , respectively (Table 3). The recovery of markers in analysed samples was assessed by comparing the area of respective peaks with the area of corresponding peaks in marker solutions.

Robustness

The robustness of the LC-MS/MS method was optimised by variations in the column temperature and the solvent flow

rate. The effect of the robustness condition on the result is shown in Table 4. The result was reported as %RSD.

Sensitivity

The proposed LC-MS/MS method, LOD and LOQ of gallic acid, corilagin acid, caffeic acid, syringic acid, rutin hydrate, ellagic acid, quercetin and morin hydrate were 0.032, 0.024, 0.022, 0.023, 0.027, 0.030, 0.024 and 0.031 $\mu\text{g/mL}$ and 0.115, 0.073, 0.065, 0.069, 0.082, 0.092, 0.072 and 0.093 $\mu\text{g/mL}$, respectively (Table 2).

Determination of Compounds 1–8 in Extracts by LC-MS/MS Analysis

The developed and validated LC-MS/MS analysis method was used to quantify components 1–8 in the PI, QI and TC extracts. The retention times of gallic acid, corilagin acid, caffeic acid, syringic acid, rutin hydrate, ellagic acid, quercetin and morin hydrate in the sample were 4.90, 12.61, 12.93, 13.17, 13.39, 13.71, 16.038 and 16.039 min, respectively as shown in Table 1, an analysis of the aqueous and hydro-alcoholic extracts of PI, QI and TC under the optimized MRM

Table 3. Precision and Recovery of the Polyphenolics Compound by the LC-MS/MS Method.

Sl. No.	Compound	Precision (%RSD)			Recovery
		Inter-day	Intra-day	Inter-analyst	
1.	Gallic acid	0.872	0.716	1.232	96.20 ± 1.16
2.	Corilagin acid	0.754	0.959	0.902	102.20 ± 1.43
3.	Caffeic acid	1.425	1.030	0.953	99.20 ± 0.91
4.	Syringic acid	0.762	0.899	0.973	103.50 ± 0.55
5.	Rutin hydrate	0.891	0.555	1.256	99.50 ± 1.45
6.	Ellagic acid	0.986	1.930	0.795	103.71 ± 0.64
7.	Quercetin	1.496	0.827	1.289	99.10 ± 1.22
8.	Morin hydrate	0.896	1.002	0.826	99.50 ± 1.19

Table 4. Robustness of the Polyphenolics Compound by the LC-MS/MS Method.

Sl. No.	Compound	(%RSD)					
		Column Temperature ($^{\circ}\text{C}$)			Flow Rate (mL/min)		
		38	40	42	0.5	0.6	0.7
1.	Gallic acid	0.168	0.110	0.129	0.815	0.701	1.021
2.	Corilagin acid	0.201	0.178	0.259	0.214	0.178	0.258
3.	Caffeic acid	0.759	0.587	0.971	0.138	0.182	0.195
4.	Syringic acid	0.049	0.037	0.070	0.173	0.224	0.258
5.	Rutin hydrate	0.512	0.416	0.789	0.091	0.082	0.091
6.	Ellagic acid	0.117	0.102	0.189	0.095	0.091	0.082
7.	Quercetin	0.154	0.109	0.149	1.027	1.003	1.045
8.	Morin hydrate	0.228	0.197	0.215	1.845	1.781	1.278

Table 5. Quantitative Estimation of Polyphenolics Compound in *P. integerrima*, *Q. infectoria* and *T. chebula* by LC-MS/MS.

Sl. No.	Compound Name	Purity (%)	<i>Pistacia integerrima</i> (µg/g)		<i>Quercus infectoria</i> (µg/g)		<i>Terminalia chebula</i> (µg/g)	
			Aqueous	Hydroalcoholic	Aqueous	Hydroalcoholic	Aqueous	Hydroalcoholic
1.	Gallic acid	99.0	54703.17 ± 699.32	43111.25 ± 465.85	106711.25 ± 951.25	84613.34 ± 589.12	49412.00 ± 491.58	37401.30 ± 426.35
2.	Corilagin acid	99.0	129.89 ± 10.25	393.51 ± 16.51	59.28 ± 7.91	97.53 ± 7.38	187.93 ± 12.81	118.15 ± 10.38
3.	Caffeic acid	99.5	3.30 ± 0.23	9.05 ± 0.94	0.07 ± 0.01	1.70 ± 0.16	0.33 ± 0.04	ND
4.	Syringic acid	95.0	3.29 ± 0.46	1.19 ± 0.22	ND	ND	ND	ND
5.	Rutin hydrate	95.0	52.83 ± 6.81	12.92 ± 1.11	0.06 ± 0.01	4.09 ± 0.31	24.52 ± 3.25	21.72 ± 2.78
6.	Ellagic acid	99.8	1407.10 ± 42.35	1004.21 ± 39.64	5105.03 ± 102.34	3522.31 ± 82.36	9897.07 ± 137.28	12398.41 ± 194.34
7.	Quercetin	97.0	115.25 ± 11.25	264.16 ± 15.62	6.36 ± 0.81	0.38 ± 0.05	ND	ND
8.	Morin hydrate	90.0	119.01 ± 12.64	277.44 ± 20.31	0.48 ± 0.06	ND	ND	ND

Note: *ND = Not detected.

Table 6. Percentage Level of Polyphenolics Compound Present in the Aqueous and Hydro-alcoholic Extracts of *Q. infectoria* and *T. chebula* with respect to *P. integerrima*.

Sl. No.	Compound Name	<i>Pistacia integerrima</i>		<i>Quercus infectoria</i>		<i>Terminalia chebula</i>	
		Aqueous	Hydro-alcoholic	Aqueous	Hydro-alcoholic	Aqueous	Hydro-alcoholic
1.	Gallic acid	100.00 ^a	100.00 ^b	195.06*** ^a	196.29*** ^b	90.31*** ^a	86.77*** ^b
2.	Corilagin acid	100.00 ^a	100.00 ^b	45.64*** ^a	24.79*** ^b	144.67*** ^a	30.03*** ^b
3.	Caffeic acid	100.00 ^a	100.00 ^b	2.21*** ^a	18.80*** ^b	10.15*** ^a	0.00
4.	Syringic acid	100.00 ^a	100.00 ^b	0.00	0.00	0.00	0.00
5.	Rutin hydrate	100.00 ^a	100.00 ^b	0.12*** ^a	31.67*** ^b	46.42*** ^a	168.05*** ^b
6.	Ellagic acid	100.00 ^a	100.00 ^b	364.29*** ^a	352.00*** ^b	707.14*** ^a	1240.00*** ^b
7.	Quercetin	100.00 ^a	100.00 ^b	5.52*** ^a	0.15*** ^b	0.00	0.00
8.	Morin hydrate	100.00 ^a	100.00 ^b	0.41*** ^a	0.00	0.00	0.00

Note: The data were indicated as mean ± SD. Results were analysed by two-way ANOVA, and the levels of significant differences among different groups were determined by the Bonferroni post-test using Graph Pad Prism ver 5.00 software. At the 95% confidence interval, $P < 0.05$ was considered as statistically significant, ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; 'a' denotes that data were compared to the aqueous extract of *P. integerrima* and 'b' denotes that data were compared to the hydro-alcoholic extract of *P. integerrima*. ns = non-significant.

conditions. Among these markers, compounds 1 (gallic acid) and 6 (ellagic acid), the major constituents of aqueous and hydro-alcoholic extracts of PI, QI and TC, were found to be present in the highest amount (Table 5). Previous studies also reported that gallic acid and ellagic acid are the important polyphenolics in PI, QI and TC.^{19,39, 42–44} The percentage levels of the marker compound present in the aqueous and hydro-alcoholic extracts of QI and TC with respect to PI are summarised in Table 6. Gallic acid and its derivatives were also reported earlier by several authors in PI, but the content of gallic acid was lower than that in our study.^{38,45} Previously, Ou et al.⁴⁰ reported and identified two polyphenolic compounds (ellagic acid and gallic acid) in QI through

HPTLC. One of the most abundant compounds, that is, ellagic acid in QI was also identified by Onal et al.⁴⁶ It is also reported that gallic acid and ellagic acid were the major polyphenolic compounds present in TC.^{47,48} Kumar et al. reported three polyphenolic compounds (gallic acid, rutin and quercetin) in TC by HPLC, but the content of these polyphenolics was lower than that in our study.⁴⁹

Conclusion

As per the best knowledge of the authors, using the selected markers, a sensitive and accurate LC-MS/MS analysis method for quality control of PI, QI and TC was developed

for the first time in this study. The approach was validated by evaluating the parameters of linearity, precision, accuracy and robustness, and it was successfully applied to sample analysis. Gallic acid and ellagic acid were found to be the most abundant polyphenolic components in the aqueous and hydro-alcoholic extracts of PI, QI and TC, as per the LC-MS/MS data. This approach exhibited that phytochemical profiling of aqueous and hydro-alcoholic extracts of PI was found similar to QI and TC, and they can be considered as substitutes for each other as per the Ayurveda texts. The developed LC-MS/MS analysis will be useful for assessing the quality of the PI, QI and TC, and further comparative efficacy evaluation study will be required to conclude the substitute.

Acknowledgements

Authors would like to show their gratitude to Dabur Research and Development Centre, for their support during the course of this research.

Summary

The developed method was accurate, precise, specific and good level of recovery.

The present study focused on liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) method development for determination of targeted polyphenolic compounds in aqueous and hydro-alcoholic extracts of PI, QI and TC.

The developed method is suitable for routine analysis of polyphenolic compounds in herbal extract.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The authors received no financial support for the research, authorship and/or publication of this article.

Statement of Informed Consent and Ethical Approval

Necessary ethical clearances and informed consent was received and obtained respectively before initiating the study from all participants.

References

- Samant SS, Pant S, Singh M, et al. Medicinal plants in Himachal Pradesh, Northwestern Himalaya, India. *Int J Biodivers Sci Manag.* 2007; 3(4): 234–51. doi: 10.1080/17451590709618177.
- Bibi Y, Zia M, Qayyum A. Review-An overview of *Pistacia integerrima* a medicinal plant species: ethnobotany, biological activities and phytochemistry. *Pak J Pharm Sci.* 2015; 28(3): 1009–13. PMID 26004708.
- Cory H, Passarelli S, Szeto J, Tamez M, Mattei J. The role of polyphenols in human health and food systems: A mini-review. *Front Nutr.* 2018; 5: 87. doi: 10.3389/fnut.2018.00087, PMID 30298133.
- Ilahi I, Khan J, Ali H, et al. Hepatoprotective and nephro-protective activities of *Pistacia integerrima* fruit extract in paracetamol intoxicated male rabbits with effect on blood cells count. *Pak J Pharm Sci.* 2019; 32(2): 817–23. PMID 31103977.
- Pant S, Samant SS. Ethnobotanical observations in the Mornaula Reserve Forest of Kumoun, West Himalaya, India. *Ethnobot Leafl.* 2010; 14: 193–217.
- Ishtiaq M, Mahmood A, Maqbool M. Indigenous knowledge of medicinal plants from Sudhanoti district (AJK), Pakistan. *J Ethnopharmacol.* 2015; 168: 201–7. doi: 10.1016/j.jep.2015.01.054, PMID 25666425.
- Ansari SH, Ali M. Analgesic and anti-inflammatory activity of tetracyclic triterpenoids isolated from *Pistacia integerrima* galls. *Fitoterapia.* 1996; 67(2): 103–5.
- Ahmad NS, Waheed A, Farman M, Qayyum A. Analgesic and anti-inflammatory effects of *Pistacia integerrima* extracts in mice. *J Ethnopharmacol.* 2010; 129(2): 250–3. doi: 10.1016/j.jep.2010.03.017, PMID 20359528.
- Joshi UP, Mishra SH. *In vitro* antioxidant activity of galls of *Pistacia integerrima*. *Pharmacologyonline.* 2009; 2: 763–8.
- Rauf A. Chemical composition and biological screening of essential oils from *Pistacia integerrima*. *Afr J Pharm Pharmacol.* 2013; 7(20): 1220–4. doi: 10.5897/AJPP2013.3467.
- Adusumalli S, Madan Ranjit P, Sankaranarayan Harish M. Antiasthmatic activity of aqueous extract of *Pistacia Integerrima* galls. *Int J Pharm.* 2013; 5(Suppl 2): 116–21.
- Ansari SH, Ali M, Qadry JS. Essential oils of *Pistacia integerrima* galls and their effect on the central nervous system. *Int J Pharmacogn.* 1993; 31(2): 89–95. doi: 10.3109/13880209309082924.
- Haq F. The critically endangered flora and fauna of district Battagram Pakistan. *Adv Life Sci.* 2012; 2(4): 118–23. doi: 10.5923/j.als.20120204.07.
- Sharma P. Threat categorization of floristic diversity of Murari Devi and surrounding areas in Mandi District of Himachal Pradesh, India. *Judge Baker Children's Center.* 2019; 5(1): 96–104. doi: 10.33980/jbcc.2019.v05i01.016.
- Sariozlu NY, Kivanc M. Gallnuts (*Quercus infectoria* Oliv. and *Rhus chinensis* Mill.) and their usage in health. In: Preedy VR, Watson RR, Patel VB, eds. *Nuts and Seeds in Health Disease Prevention.* Academic Press; 2011: 505–11.
- Rajopadhye AA, Upadhye AS. Botanical and phytochemical standardization of *Fumaria vaillantii* Loisel. *Indian J Nat Prod Resour.* 2011; 2(3): 369–74.
- Ikram M, Nowshad F. Constituents of *Quercus infectoria*. *Planta Med.* 1977; 31(3): 286–7. doi: 10.1055/s-0028-1097531, PMID 866492.
- Hamid H, Kaur G, Abdullah ST, Ali M, Athar M, Alam MS. Two new compounds from the falls of *Quercus infectoria* with nitric oxide and superoxide inhibiting ability. *Pharm Biol.* 2005; 43(4): 317–23. doi: 10.1080/13880200590951711, PMID 28925837.
- Shrestha S, Kaushik VS, Eshwarappa RSB, Subaramaihha SR, Ramanna LM, Lakkappa DB. Pharmacognostic studies of insect gall of *Quercus infectoria* Olivier (Fagaceae). *Asian Pac J Trop Biomed.* 2014; 4(1): 35–9. doi: 10.1016/S2221-1691(14)60205-7, PMID 24144128.
- Evans WC. Pharmacopoeial and related drugs of biological origin; Part 5. Trease and Evans' Pharmacognosy. W. B. Saunders; 2009.
- Hwang JK, Kong TW, Baek NI, Pyun YR. α -glycosidase inhibitory activity of hexagalloylglucose from the galls of *Quercus*

- infectoria*. *Planta Med.* 2000; 66(3): 273–4. doi: 10.1055/s-2000-8569, PMID 10821056.
22. Dar MS, Ikram M. Studies on *Quercus infectoria*; isolation of syringic acid and determination of its central depressive activity. *Planta Med.* 1979; 35(2): 156–61. doi: 10.1055/s-0028-1097197, PMID 419182.
 23. Kaur G, Hamid H, Ali A, Alam MS, Athar M. Antiinflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*. *J Ethnopharmacol.* 2004; 90(2–3): 285–92. doi: 10.1016/j.jep.2003.10.009, PMID 15013194.
 24. Basri DF, Tan LS, Shafiei Z, Zin NM. *In vitro* antibacterial activity of galls of *Quercus infectoria* Olivier against oral pathogens. *Evid Based Complement Alternat Med.* 2012; 2012: 632796. doi: 10.1155/2012/632796, PMID 22203875.
 25. Basri DF, Fan SH. The potential of aqueous and acetone extracts of galls of *Quercus infectoria* as antibacterial agents. *Indian J Pharmacol.* 2005; 37(1): 26–9. doi: 10.4103/0253-7613.13851.
 26. Karmegam N, Karuppusamy S, Prakash M, Jayakumar M, Rajasekar K. Antibacterial potency and synergistic effect of certain plant extracts against food-borne diarrheagenic bacteria. *Int J Pharm Biomed Res.* 2008; 2(2): 88–93.
 27. Hussein G, Miyashiro H, Nakamura N, Hattori M, Kakiuchi N, Shimotohno K. Inhibitory effects of Sudanese medicinal plant extracts on hepatitis C virus (HCV) protease. *Phytother Res.* 2000; 14(7): 510–6. doi: 10.1002/1099-1573(200011)14:7<510, PMID 11054840.
 28. Baharuddin NS, Abdullah H, Abdul Wahab WN. Anti-*Candida* activity of *Quercus infectoria* gall extracts against *Candida* species. *J Pharm Bioallied Sci.* 2015; 7(1): 15–20. doi: 10.4103/0975-7406.148742, PMID 25709331.
 29. Tayel AA, El-Sedfy MA, Ibrahim AI, Moussa SH. Application of *Quercus infectoria* extract as a natural antimicrobial agent for chicken egg decontamination. *Rev Argent Microbiol.* 2018; 50(4): 391–7. doi: 10.1016/j.ram.2017.12.003, PMID 29691109.
 30. Kaur G, Athar M, Alam MS. *Quercus infectoria* galls possess antioxidant activity and abrogates oxidative stress-induced functional alterations in murine macrophages. *Chem Biol Interact.* 2008; 171(3): 272–82. doi: 10.1016/j.cbi.2007.10.002, PMID 18076871.
 31. Ahad A, Mujeeb M, Ahsan H, Siddiqui WA. Nephroprotective potential of *Quercus infectoria* galls against experimentally induced diabetic nephropathy in rats through inhibition of renal oxidative stress and TGF- β . *Anim Cells Syst.* 2016; 20(4): 193–202. doi: 10.1080/19768354.2016.1207703.
 32. Rehman MU, Tahir M, Ali F, et al. Chemopreventive effect of *Quercus infectoria* against chemically induced renal toxicity and carcinogenesis. *Int J Drug Dev Res.* 2012; 4(2): 336–51.
 33. Bag A, Bhattacharyya SK, Chattopadhyay RR. The development of *Terminalia chebula* Retz. *Asian Pac J Trop Biomed.* 2013; 3(3): 244–52. doi: 10.1016/S2221-1691(13)60059-3, PMID 23620847.
 34. Klika KD, Saleem A, Sinkkonen J, et al. The structural and conformational analyses and antioxidant activities of chebulinic acid and its thrice-hydrolyzed derivative, 2,4-chebuloyl- β -D-glucopyranoside, isolated from the fruit of *Terminalia chebula*. *Arkivoc*; 2004(7): 83–105. doi: 10.3998/ark.5550190.0005.708.
 35. Rubab I, Ali S. Dried fruit extract of *Terminalia chebula* modulates the immune response in mice. *Food Agric Immunol.* 2016; 27(1): 1–22. doi: 10.1080/09540105.2015.1055554.
 36. Majed F, Nafees S, Rashid S, et al. *Terminalia chebula* attenuates DMBA/croton Oil-Induced oxidative stress and inflammation in Swiss albino mouse skin. *Toxicol Int.* 2015; 22(1): 21–9. doi: 10.4103/0971-6580.172252, PMID 26862256.
 37. Choudhari DD, Kukarni YR, Visave PB. Abhava pratinidhi dravya: A comparative phytochemical study of Bharangi and Kantakari. *Int J Ayurveda Res.* 2021; 12(3): 588–92.
 38. Zahoor M, Zafar R, Rahman NU. Isolation and identification of phenolic antioxidants from *Pistacia integerrima* gall and their anticholine esterase activities. *Heliyon.* 2018; 4(12): e01007. doi: 10.1016/j.heliyon.2018.e01007, PMID 30623127.
 39. Singh A, Bajpai V, Kumar S, Kumar B, Srivastava M, Rameshkumar KB. Comparative profiling of phenolic compounds from different plant parts of six *Terminalia* species by liquid chromatography–tandem mass spectrometry with chemometric analysis. *Ind Crops Prod.* 2016; 87: 236–46. doi: 10.1016/j.indcrop.2016.04.048.
 40. Ou L, He Q, Ji Z, Li K, Tian S. Quantitative high-performance thin-layer chromatographic analysis of three active compounds in gall of *Quercus infectoria* Olivier (Fagaceae) and use of thin-layer chromatography–2,2-diphenyl-1-picrylhydrazyl to screen antioxidant component. *J Planar Chromatogr Mod TLC.* 2015; 28(4): 300–6. doi: 10.1556/1006.2015.28.4.6.
 41. Abdullah AR, Hapidin H, Abdullah H. Phytochemical analysis of *Quercus infectoria* galls extracts using FTIR, LC-MS and MS / MS analysis. *Res J Biotechnol.* 2017; 12: 55–61.
 42. Alhumaydhi FA, Rauf A, Rashid U, et al. *In vivo* and *in silico* studies of flavonoids isolated from *Pistacia integerrima* as potential antidiarrheal agents. *ACS Omega.* 2021; 6(24): 15617–24. doi: 10.1021/acsomega.1c00298, PMID 34179606.
 43. Seraglio SKT, Valese AC, Daguer H, et al. Development and validation of a LC-ESI-MS/MS method for the determination of phenolic compounds in honeydew honeys with the diluted-and-shoot approach. *Food Res Int.* 2016; 87: 60–7. doi: 10.1016/j.foodres.2016.06.019, PMID 29606249.
 44. Afshari AR, Sadeghnia HR, Mollazadeh H. A review on potential mechanisms of *Terminalia chebula* in Alzheimer's disease. *Adv Pharmacol Sci.* 2016; 2016: 8964849. doi: 10.1155/2016/8964849, PMID 26941792.
 45. Balkrishna A, Verma S, Sharma P, Tomer M, Srivastava J, Varshney A. Comprehensive and rapid quality evaluation method for the ayurvedic medicine Divya-Swasari-Vati using two analytical techniques: UPLC/QToF MS and HPLC-DAD. *Pharmaceuticals (Basel).* 2021; 14(4): 297. doi: 10.3390/ph14040297, PMID 33801579.
 46. Onal A, Sari A, Soylak M. Ellagic acid from gallnut (*Quercus infectoria*): Extraction and determination of its dyeing conditions for natural fibres. *J Sci Ind Res.* 2005; 64: 491–5.
 47. Jadhav PD, Laddha KS. Estimation of gallic acid and ellagic acid from *Terminalia chebula* Retz. *Indian Drugs.* 2004; 41(4): 200–6.
 48. Choudhary RAK, Manivannan E, Rajeswari P, Sivasankari V, Chandrashekar R AK, Ravi I. Determination and evaluation of ellagic acid in *Terminalia chebula* fruit extract by RP-HPLC. *Pharmacology Online.* 2021; 2: 598–606.
 49. Kumar A, Lakshman K, Jayaveera KN, Tripathi SNM, Satish KV. Estimation of gallic acid, rutin and quercetin in *Terminalia chebula* by HPTLC. *Jordan J Pharm Sci.* 2010; 3(1): 63–8.