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Pattern profiling of the herbal preparation picroliv using liquid chromatography–tandem mass spectrometry☆

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Abstract

At present, the construction of chromatographic fingerprints of complex herbal preparations in combination with mass spectrometry plays an important role in their development and standardization as potential therapeutic agents. Picroliv, an extract from roots and rhizomes of *Picrorhiza kurroa*, is a herbal hepatoprotective developed by CDRI. We report for the first time pattern profiling of various constituents of picroliv along with a precise and accurate method to estimate relative concentration of major components in the preparation by liquid chromatography–tandem mass spectrometry. In total, 27 components could be detected in multiple reaction monitoring (MRM) mode out of which fourteen could be quantified in terms of their relative concentration. Seven components were structurally correlated and confirmed based on the fragmentation pattern and information available in literature. The detection was carried out using MRM in negative ionization mode with analytes quantified from the summed total ion value of their most intense molecular ion transitions. The separation of various components was achieved using a gradient elution on RP-18 column with acetonitrile and Milli-Q water as mobile phase at a flow rate of 1.0 ml/min. The method was validated in terms of linearity, accuracy and precision (within- and between-assay variation) for 5 days. Linearity range was different for various components depending upon their sensitivity and abundance in the herbal preparation. Within- and between-assay accuracy (%bias) and precision (%R.S.D.) were within acceptable limits. The method was successfully applied to detect and determine relative concentrations of various components in two different batches of picroliv.

Keywords: Picroliv; *Picrorhiza kurroa*; Fingerprinting; Validation; Pharmaceutical analysis; Picrosides; Kutkoside

1. Introduction

Herbal medicines have a long history of their usage for treatment of various clinical situations [1]. Recent WHO guidelines state "historical use of a substance is a valid way to document safety and efficacy in the absence of scientific evidences to the contrary [2]." However, instances of varying efficacy and toxicity with many commercially available herbal preparations have raised doubts on effectiveness and safety reputation of herbal preparations [3–5]. In practice, herbal preparation standardizations are based on one or two major chemical constituents. Such standardization however, still has span for variation due to presence of multiple constituents, which may be responsible for therapeutic effect. They work synergistically and hence cannot be separated into active parts. Therefore, it is necessary to define maximum possible phytochemical constituents of these botanical extracts to ensure the reliability and repeatability in clinical and pharmacological research and also to understand bioactivities and possible adverse effects [6]. At this juncture, it may not be important to characterize every constituent of herbal preparation but number of constituents and their relative concentrations (Pat profiling) can serve as a rational means for standardization of biologically active herbal preparation.

With the advent of mass spectrometry (MS), methods based on liquid chromatography tandem mass spectrometry (LC–MS–MS) can help in pat profiling and also structural elucidation of different components in a herbal preparation in combination with literature reports [7–11]. Picroliv preparation (PP) has been developed by Central Drug

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Research Institute, Lucknow, from the roots and rhizomes of *Picrorhiza kurroa* Royle (Scrophulariaceae) [12]. It forms a major ingredient of many Ayurvedic preparations prescribed in the treatment of several ailments of liver and spleen, fever and asthma [13–17]. PP has shown excellent hepatoprotective activity and immunomodulatory action in number of laboratory studies [18–21]. At present it is standardized based on picroside I and kutkoside [22–29].

This communication presents for the first time detection of 27 components and development and validation of an assay method with relative quantitation of various components in PP. Of these, seven components could be identified based on the fragmentation pattern and literature reports. The method was successfully applied to determine relative concentrations of different components in two batches of PP.

2. Experimental

2.1. Herbal materials and chemicals

Three batches of PP were obtained from Pharmaceutics Division of CDRI, Lucknow, India. Metronidazole (IP grade) was obtained as a generous gift from Unichem Laboratories, Mumbai, India. Acetonitrile (HPLC grade), sodium chloride and potassium chloride (analytical reagent grade) were obtained from Qualigens Fine Chemicals, Mumbai, India. Ammonium acetate (GR grade) was procured from E. Merck India, Mumbai, India. Purified deionised water was obtained from a Milli-Q (Millipore, USA) water purification system.

2.2. Liquid chromatography

A Perkin-Elmer Series 200 HPLC system (Perkin-Elmer, USA) with auto injector was used to deliver the mobile phase [Milli-Q water (solvent A) and acetonitrile–Milli-Q water (50:50, %v/v; solvent B)] at a flow rate of 1 ml/min in gradient elution mode. The gradient started with pump supplying solvents A and B at the rate 85 and 15%, respectively. Solvent A decreased to 7% within 50 min following B-2 curve and then increased linearly to 85% by 55 min and maintained till 60 min. Chromatographic separations were achieved on Discovery RP-18 column (150 mm × 4.6 mm I.D., 5 μm particle size) preceded with guard column packed with the same material. The samples (50 μl) were injected onto the mass spectrometer.

2.3. Mass spectrometry analysis

The API-4000 (Applied Biosystems, Toronto, Canada) mass spectrometer was operated using a standard ESI source coupled with the LC separation system. Analyst software (Version 1.3.2, Applied Biosystems, Toronto, Canada) was used for the control of equipment, acquisition and data analysis.

MS parameters were optimized by constant infusion of a known concentration of PP. MS scan was performed in both positive and negative ion mode and declustering potential was optimized. For MS–MS optimization, the optimized declustering potentials were used with nitrogen as the collision gas to obtain prominent product ions. Information dependent acquisition (IDA) was utilized to gather information of molecular ion and their products in a single LC–MS run.

2.4. Analytical standards and quality control samples

PP (1 mg/ml) and internal standard (IS) metronidazole (100 μg/ml) were prepared individually in Milli-Q water. Working stock solutions of PP (WS 1, 57.6 μg/ml) and IS (WS 2, 10 μg/ml) were prepared by further dilutions of respective stock solutions in Milli-Q water. Analytical standards in a concentration range of 0.030–3.84 μg/ml of PP were prepared from WS 1 in Milli-Q water. IS was added to each analytical standard to achieve a concentration of 0.5 μg/ml. Quality control (QC) samples at low (L1, 0.03 μg/ml; L2, 0.06 μg/ml), medium (M, 0.48 μg/ml) and high concentration (H, 3.84 μg/ml) were prepared in triplicate each day.

2.5. Method validation

The validation of LC–MS–MS method included within- and between-run accuracy and precision determination of quality control samples in triplicate for five different days. MRM in negative ion mode was utilized for quantitation.

2.6. Limit of detection (LOD) and lowest limit of quantitation (LLOQ)

The LOD of assay method for different components in PP was the lowest quantity in sample corresponding to three times the baseline noise (S/N > 3) and LLOQ was defined as the relative concentration with S/N > 10.

2.7. Specificity, accuracy and precision

The specificity of the method was defined as non-interference in the regions of interest, for quantification of various components of PP. Within- and between-assay precision (%R.S.D.) was determined by subjecting the data to one-way analysis of variance (ANOVA). Within- and between-assay accuracy was determined by calculating %bias from the theoretical concentration using the following equation:

$$\%\text{bias} = [(\text{observed concentration} - \text{nominal concentration})/\text{nominal concentration}] \times 100.$$

2.8. Application of the method for analysis of different batches of PP

The method was applied to determine various components and their relative concentrations in two different batches

Table 1
Molecular ions detected in full scan mode

Code	Name (M_r)	$[M + Na]^+$	$[M - H]^-$	t_R (min)
A	Picroside IV (508)	531	507	26.7
B	Picroside III (538)	561	537	28.1
C	Kutkoside (512)	535	511	29.6
D	Picroside I (492)	515	491	34.9
E	6-Feruloyl catalpol/ minecoside (538)	561	537	35.6
F	6-Feruloyl catalpol/ minecoside (538)	561	537	36.2
G	Scroside B (668)	691	667	36.6

(batch-2 and -3) of PP. Batch 1, which was found biologically active, was taken as standard for test batches. A known concentration of PP (1.92 μg/ml) from two batches was prepared together with IS in triplicate and relative concentration of various components (mean ± S.D.) in test batches was determined.

3. Results and discussion

3.1. Optimization of LC and MS conditions

LC conditions were optimized to the conditions described in experimental section using Milli-Q water (solvent A) and acetonitrile: Milli-Q water (50:50, v/v; solvent B) at 1.0 ml/min in gradient elution. Out of different columns tried, best results were obtained on discovery RP-18 column (150 mm × 4.6 mm, 5 μm) preceded with guard. MS conditions for picroside I and kutkoside were optimized by constant infusion while full scan MS with LC gradient was used to identify other components of PP in positive and negative ion modes. Information from full scan MS was utilized to build an IDA method, which provided data of precursor and product ions in a single LC–MS run.

Picroside I (MW 492) and kutkoside (MW 512) in positive ion mode gave sodium adduct at *m/z* 515 and 535, respectively. Protonated and potassium adducts, though weak in intensity were also observed for picroside I and kutko-

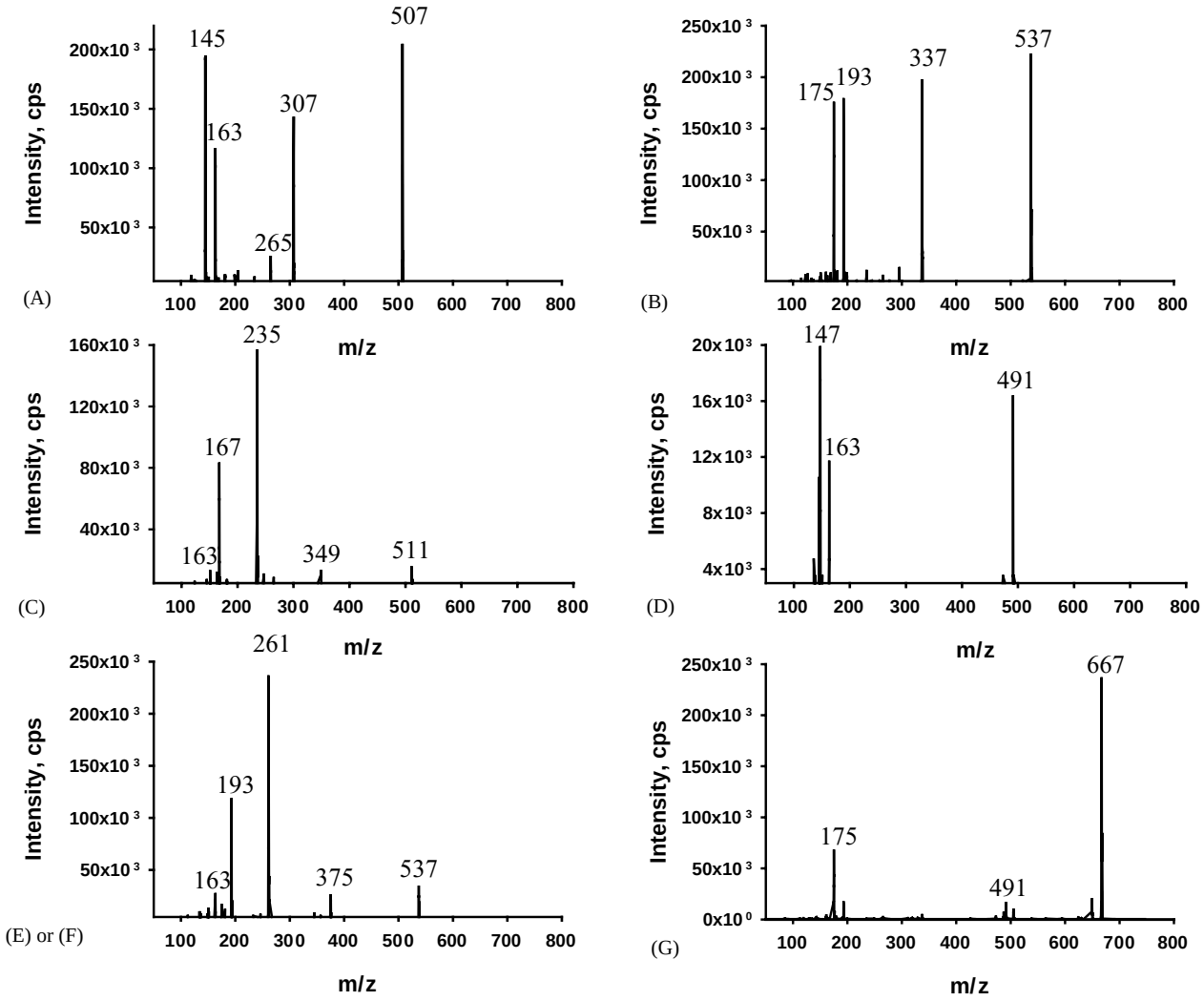


Fig. 1. Product ion spectra of A (*m/z* 507), B (*m/z* 537), C (*m/z* 511), D (*m/z* 491), E or F (*m/z* 537), and G (*m/z* 667) in negative ion mode.

Table 2
MRM conditions for known components

Analyte code	$[M - H]^-$	Product ion(s)	Declustering potential (V)	Collision energy (eV)	Linearity (μg/ml)
A	507	145	90	35	0.030–3.84
		307			
B	537	193	80	35	0.120–3.84
		337		30	
C	511	167	95	32	0.030–3.84
		235			
D	491	147	60	25	0.030–3.84
		163		14	
E	537	193	95	35	0.030–3.84
		261		30	
F	537	193	95	35	0.030–3.84
		261		30	
G	667	175	90	45	0.120–3.84
		491			

side at *m/z* 493, 531 and 513, 551, respectively. Owing to division of signal between sodium, potassium and hydrogen ions, the sensitivity was compromised. Then, infusion in negative ion mode was tried and strong signals of picroside I and kutkoside as $[M - H]^-$ were observed at *m/z* 491 and 511, respectively, favoring negative ion mode for detection of these two components. Declustering potentials for picroside I and kutkoside were optimized to −60 V and −95 V, respectively.

For other molecular entities, injection of PP (50 μg/ml) was given through column in a scan range of 100–800 in positive and negative ion mode with different declustering potentials. The results of positive and negative ion modes indicated five additional components apart from picroside I and kutkoside (Table 1). The intensity of molecular ion peak for most of the components was greater in negative ion mode. In negative and positive ion mode, three components with molecular mass 538 were detected as separate distinct peaks at 28.1, 35.6, 36.2 min indicating possibility of isomers in PP.

3.2. Optimization of MS–MS conditions

IDA approach was utilized to get MS–MS transitions along with precursor ions in a single LC–MS run. In total, 27 components with their product ion spectra could be observed of which, seven components matched with the literature information. The product ion spectra of seven components of PP (A–G) in negative ion mode are given in Fig. 1. Utilizing this information, an MRM method for quantitation was developed and the collision energy (CE) was optimized for different transitions by giving on column injections. Nebulizing gas (GS 1), turbo gas (GS 2), curtain gas and temperature were set to 20, 60, and 16 psi and 600 °C, respectively (1 psi = 6894.76 Pa). Wherever two prominent fragments were obtained, they were incorporated in MRM method to increase the sensitivity of the method and their summed ions were used for quantification otherwise a single prominent ion was used. Metronidazole was used as IS eluting at 4.5 min under chromatographic conditions described earlier and was monitored using MRM in positive ion mode. The IS provided linear peak area ratios for most

Table 3
Accuracy (bias) and precision (R.S.D.) of validated components

Analyte code	Concentration (μg/ml)	Bias (%)		R.S.D. (%)	
		Intra-batch	Inter-batch	Intra-batch	Inter-batch
A	0.03	5.0	4.1	3.6	3.5
	0.06	0.8	3.9	2.8	7.4
	0.48	−4.4	3.0	5.7	8.9
	3.84	−3.6	−1.3	4.2	1.0
B[a]	0.12	1.4	0.5	1.3	2.3
	0.48	−1.9	−2.7	1.7	2.9
	3.84	0.8	2.8	2.1	4.6
C	0.03	1.6	1.1	2.2	6.1
	0.06	0.7	−0.4	1.9	4.3
	0.48	−0.6	0.8	5.1	3.5
	3.84	−1.4	−2.7	3.3	4.0
D	0.03	3.3	1.7	2.9	8.0
	0.06	2.4	2.6	3.7	2.4
	0.48	9.8	3.6	9.4	4.4
	3.84	−3.4	−3.4	5.4	7.1
E	0.03	1.9	2.1	3.9	6.0
	0.06	0.3	−1.2	3.2	8.3
	0.48	0.9	1.9	1.9	2.4
	3.84	0.3	0.2	1.8	5.7
F	0.03	0.2	−3.9	2.4	7.8
	0.06	−0.03	−3.3	3.2	8.7
	0.48	1.5	3.0	2.5	2.7
	3.84	−4.5	−1.1	5.5	4.1
G[a]	0.12	0.4	−0.6	4.1	5.1
	0.48	0.5	−0.4	4.5	6.9
	3.84	−3.4	0.7	3.0	1.0

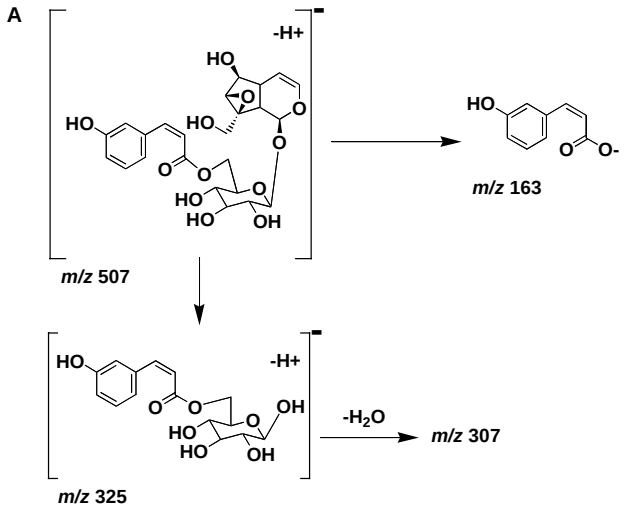
[a] For B and G, quality control sample at 0.12 μg/ml was used as LQC (low quality control).

analytes throughout the analytical range. The optimized MRM conditions for quantitation are given in Table 2.

3.3. Fragmentation of various components in negative ion mode

The structural information for various detected components (A–G) were obtained from literature [22,25,26,30–32]. The proposed fragmentation pattern for these components in negative ionization mode is illustrated in Figs. 2–4.

On MS–MS analysis, component A indicated a fragment with *m*/*z* 163 which could be formed due to breakage of glucosidic linkage at 6-C of glucose (Fig. 2) [33,34]. Fragment with *m*/*z* 325 may be formed by loss of iridoid moiety from the parent ion, which on water loss resulted in fragment with *m*/*z* 307 [35]. Component C yielded a fragment with *m*/*z* 167 which is likely to be formed due to breakage from iridoid nucleus (Fig. 3). Fragment with *m*/*z* 349 might be formed due to breakage of glucosidic linkage with aglycone [33,34]. Component D resulted in fragment with *m*/*z* 147 on breakage of glucosidic linkage at 6-C of glucose. Component G yielded fragments with *m*/*z* 491 and 193 due to breakage of glucosidic linkages at 1 and 6-C of inner glucose molecule, respectively. Fragment *m*/*z* 193 on water loss resulted in fragment with *m*/*z* 175 (Fig. 4). Components B, E and F, showed MS signals at *m*/*z* 537 in negative ion mode (Figs. 2 and 4). However, different LC elution times (Table 1), clearly demonstrate that they are different chemical entities [11,30,31]. Of these, two components (E and F) had similar MS–MS spectra whereas the third component (B) showed some different fragments. The observed difference in the fragments is logical as the position of feruloyl moiety in B is different than that in E and F. Component B showed three fragments with *m*/*z* 337, 193 and 175 (Fig. 2). Removal of iridoid moiety resulted in fragment with *m*/*z* 355 which on neutral loss of water gave a fragment with *m*/*z* 337.



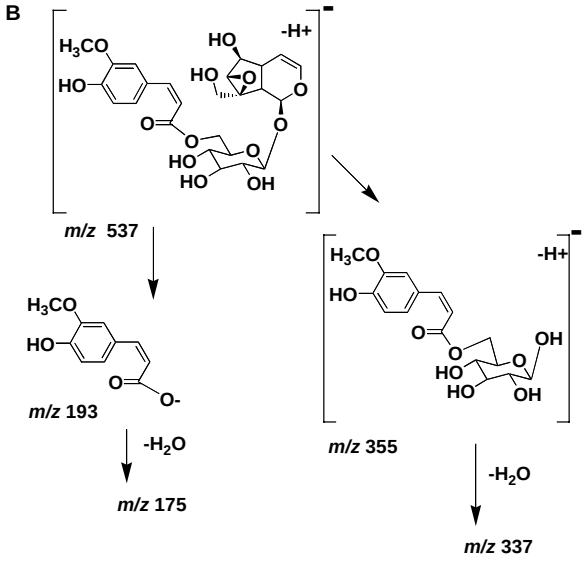
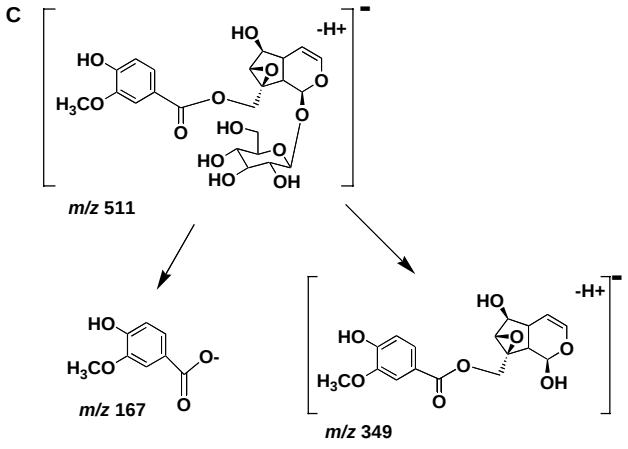


Fig. 2. Proposed fragmentation pathways for A and B in negative ionization mode.



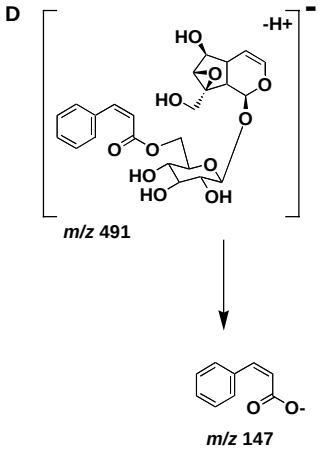


Fig. 3. Proposed fragmentation pathways for C and D in negative ionization mode.

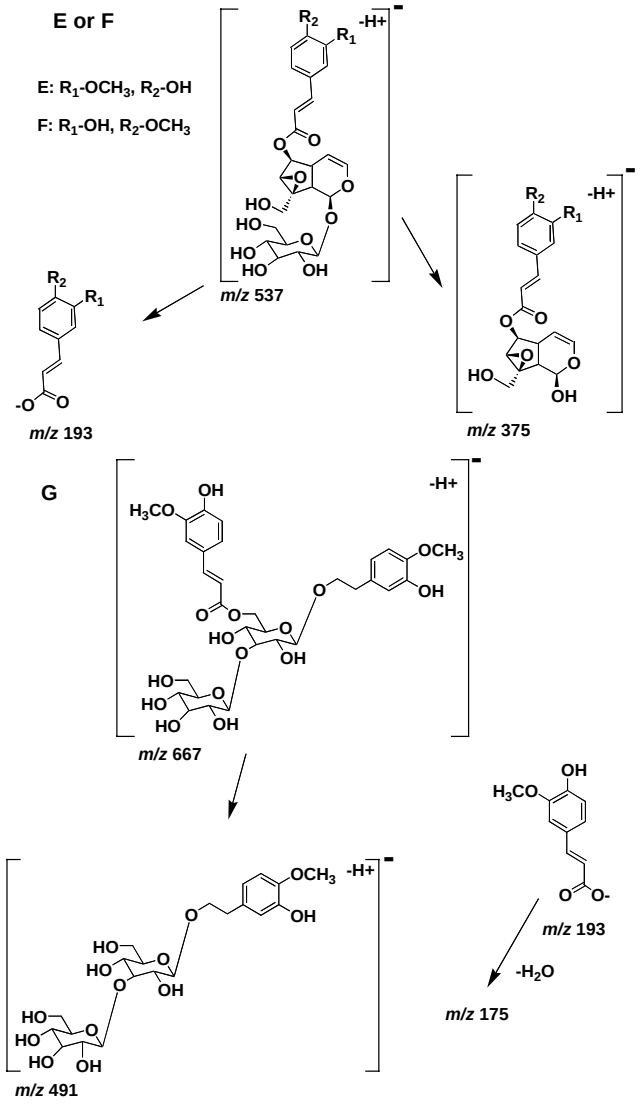


Fig. 4. Proposed fragmentation pathways for E, F and G in negative ionization mode.

Fragment with *m*/*z* 193 correspond to negative ion of feruloyl moiety, which on loss of water resulted in a fragment with *m*/*z* 175. Fragment with *m*/*z* 337 is characteristic for component B as the feruloyl moiety is linked directly to glucose. This fragment cannot be formed either from E or F due to difference in the linkage of feruloyl moiety. From components E and F, fragments with *m*/*z* 375 and 193 were formed. Fragment with *m*/*z* 375 could be formed due to breakage of glucosidic linkage (Fig. 4). Fragment with *m*/*z* 193 is formed by selective removal of feruloyl moiety from iridoid nucleus.

3.4. Method validation

3.4.1. Linearity and analytical standard range

The peak area ratios of A–G were found to be linear in their respective analytical range (Table 3). The peak area ratio of different components to IS varied linearly with concentration over the range 0.030–3.84 μg/ml. The analytical model was selected based on the analysis of data by least square linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$ and $1/x^2$). The best fit for the analytical curve could be achieved by a linear equation of $y = mx + c$ and $1/x$ weighting factor.

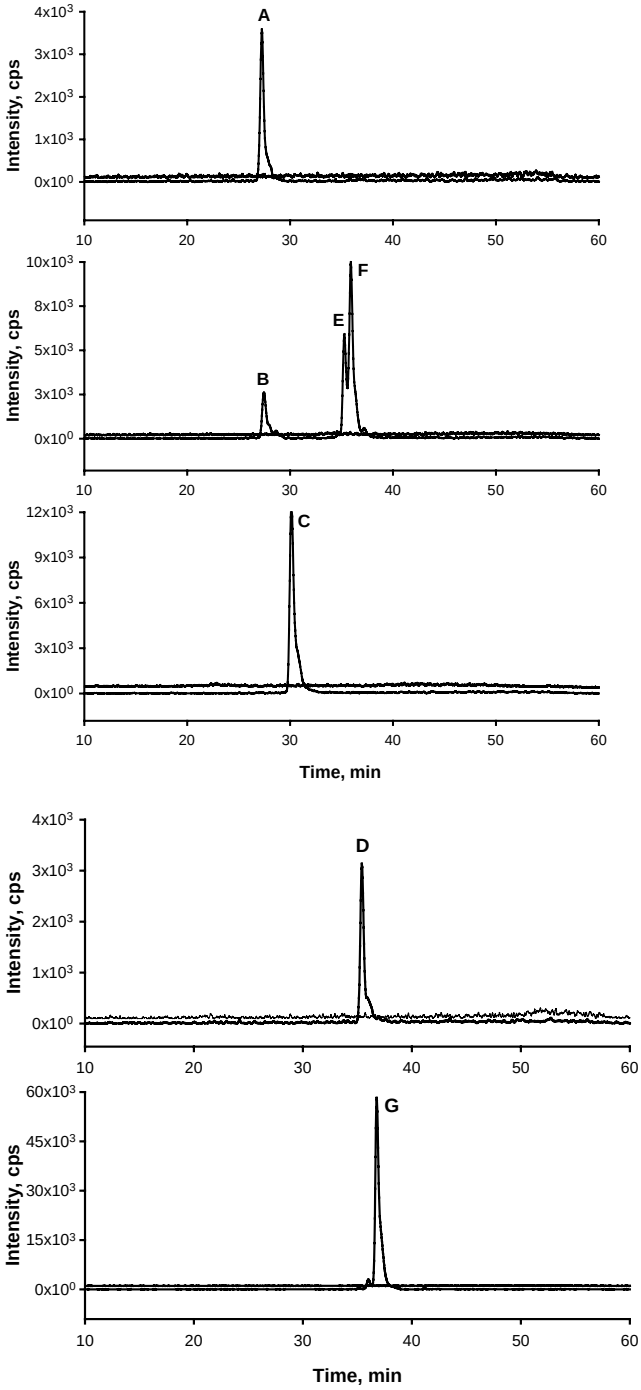


Fig. 5. Representative chromatograms of A–G at their respective LLOQ overlaid with blank in their respective MRM modes.

Table 4
Details of various components [detected (+)/could not be detected (−)] and their relative concentrations (mean ± S.D.) in two different batches of PP at a relative concentration of 1.92 μg/ml

Number	Name (M_r)	Batch 1 (reference)	Batch 2 (test) mean ± S.D.	Batch 3 (test) mean ± S.D.
1	Picroside IV (508)	+	+ (1.36 ± 0.06)	+ (2.54 ± 0.14)
2	Picroside III (538)	+	+ (0.97 ± 0.07)	+ (2.10 ± 0.19)
3	Kutkoside (512)	+	+ (0.84 ± 0.04)	+ (1.67 ± 0.06)
4	Picroside I(492)	+	+ (2.06 ± 0.05)	+ (1.87 ± 0.07)
5	6-Feruloyl catalpol (538)/minecoside(538)	+	+ (1.64 ± 0.09)	+ (2.28 ± 0.09)
6	6-Feruloyl catalpol (538)/minecoside(538)	+	+ (2.94 ± 0.19)	+ (3.01 ± 0.24)
7	Scroside-B (668)	+	+ (2.91 ± 0.13)	+ (2.99 ± 0.38)
8	330	+	+ (0.63 ± 0.04)	+ (1.44 ± 0.02)
9	566	+	+ (2.31 ± 0.19)	+ (1.98 ± 0.17)
10	474	+	+ (1.17 ± 0.01)	+(1.78 ± 0.06)
11	566	+	+ (0.33 ± 0.00)	+ (0.39 ± 0.00)
12	510	+	+ (0.81 ± 0.02)	+ (3.77 ± 0.04)
13	444	+	+ (3.64 ± 0.04)	+ (1.31 ± 0.02)
14	266	+	+	+
15	491	+	−	+
16	266	+	+	+
17	296	+	−	−
18	482	+	−	−
19	482	+	−	−
20	510	+	+	+
21	476	+	+	+
22	510	+	+	+
23	474	+	+	+
24	712	+	−	−
25	658	+	−	−
26	586	+	−	−
27	566	+	+	+

3.4.2. Specificity, accuracy and precision

Representative chromatograms of A–G at their LLOQ overlaid with blank in their respective MRM modes are given in Fig. 5 indicating absence of interference. The overall %bias and %R.S.D. at four different concentrations is presented in Table 3. The variations were less than ±10% at all concentration levels for A–G indicating that the analytical method is accurate and precise.

3.5. Application of the method

The validated method was employed for analysis of two different batches of PP. The results are summarized in Table 4. The number of components observed was different in both batches. Moreover, relative concentrations of these components varied, which are expected due to batch-to-batch variations commonly observed in herbal preparations.

4. Conclusion

Pat profiling of PP using LC–MS–MS indicated the presence of five additional known components apart from picroside I and kutkoside. The MRM method was developed and validated for relative quantification of these components over a concentration range of 0.030–3.840 μg/ml. The method was used to estimate relative concentrations of different components in two batches of PP. This is a first attempt of its kind for standardization of complex herbal preparations using tandem mass spectrometry. More detailed work in terms of component identification and analysis is still needed.

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