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Simultaneous quantification of protoberberine and aporphine alkaloids in different plant parts of *Coscinium fenestratum* (Gaertner) Colebr. By liquid chromatography-hybrid triple quadrupole/linear ion trap mass spectrometer

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Abstract
Coscinium fenestratum is extensively used as medicinal plant in traditional medicine to treat variety of ailments. Protoberberine and aporphine alkaloids are reported the major components in stem and root of this plant. In present study, quantification of eight bioactive alkaloids in different plant parts of *C. fenestratum* was done using ultra-performance liquid chromatography coupled to hybrid triple quadrupole/linear ion trap mass spectrometry in multiple reactions monitoring acquisition. The analytical procedure was validated; obtaining precision and recoveries of the samples ranging from 0.40-3.1% and 0.59-3.17% (98-101%), respectively (expressed as relative standard deviations, RSDs). Calibration curves for all the eight analytes provided optimum linear detector response (with $R^2 \geq 0.9988$) over the concentration range of 0.5-1000 ng/mL. Finally, the method was applied to different plant parts of *C. fenestratum*, and observed that root part had higher level of total content whereas flower part had the lowest level of total content of eight analytes.

Keywords: *Coscinium fenestratum*, Daruharidra, Quantification, UPLC-ESI-MS/MS, Alkaloids.

1. Introduction
Coscinium fenestratum (Gaertner) Colebr. is a medicinal plant belonging to family Menispermaceae, and it has been used as an herbal medicine 'Daruharidra' in the Indo-China region, south India, and Sri Lanka for a long time [1,2]. This plant has been used in the treatment of several ailments such as diarrhea, inflammation, ulcers, skin disease, and diabetes mellitus [3-6]. Several pharmacological activities of the plant extracts have been attributed including anti-bacterial, anti-fungal, anti-oxidant, anti-diabetic, and hypotensive activities [7-10]. Mainly quaternary and tertiary protoberberine alkaloids *viz* berberine, palmatine, tetrahydropalmatine, jatrorrhizine, etc. have been isolated previously [11-14]. Among these, berberine has been reported to be the major and active constituent in *C. fenestratum*.
Several analytical methods are reported to measure the major component of this plant i.e. berberine. These reported methods are based on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) [14-16]. However, the analytes in these literatures were identified only by their retention time (tR), UV spectra and TLC system. Compared with these analytical methods, combination of the ultra-performance liquid chromatography and hybrid triple quadrupole/linear ion trap mass spectrometry (UPLC-QqQ_{LIT}-MS) analytical method in multiple reactions monitoring (MRM) acquisition is a more powerful approach which is used in pharmaceutical research and for quality control in herbal medicine [14]. This method quickly quantifies multiple components in a crude extract due to its rapid separation power, greatest sensitivity and high specificity. In this paper, an UPLC-ESI-MS/MS method is used for simultaneously determination of eight bioactive alkaloids in different plant parts such as leaf, root, stem, and flower of *C. fenestratum* using UPLC-QqQ_{LIT}-MS operated in MRM acquisition.

2. Materials and methods
2.1 Materials
AR grade ethanol, purchased from Merck Millipore (Darmstadt, Germany), was used in the preparation of ethanolic extract. LC-MS grade methanol, acetonitrile and formic acid,

purchased from Sigma-Aldrich (St. Louis, MO, USA), were used in mobile phase and sample preparation throughout the LC-MS studies. Ultra-pure water, obtained from Direct-Q system (Millipore, Billerica, MA, USA), was used throughout the analysis. Standard compounds of berberine, palmatine, jatrorrhizine, magnoflorine, tetrahydroberberine, tetrahydropalmatine, glaucine and isocorydine were purchased from Shanghai Tauto Biotech Co., Ltd (Shanghai, China). The structures of these standard compounds are shown in Fig. 1. Plant material was collected from the Palode, Kerala, India and voucher herbarium specimen (No. TBGT 66456 for *C. fenestratum*) was deposited in the Herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala (JNTBGRI).

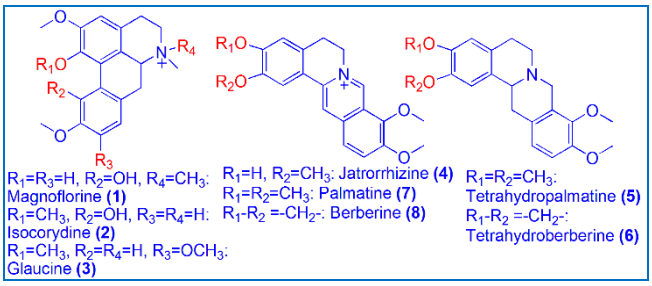

Fig 1: Structures of eight bioactive compounds from *Coscinium fenestratum*

2.1 Extraction and Sample Preparation

25 g powder of each dried plant parts were extracted with 250 mL ethanol (100%) in an ultrasonic water bath for 15 min and then kept under room temperature. After 24 h, the extracts were filtered through filter paper (Whatman No. 1) and residues were re-extracted three times with fresh solvent following the same procedure. The combined filtrates of each sample were evaporated to dryness under reduced pressure at 20-50 kPa and temperature at 40°C using a Buchi rotary evaporator (Flawil, Switzerland). Stock solutions (1 mg/mL) of each sample were prepared in methanol and filtered through a 0.22-µm PVDF membrane (Merck Millipore, Darmstadt, Germany).

2.2 Preparation of standard solutions

Stock solutions of berberine, palmatine, jatrorrhizine, tetrahydroberberine, tetrahydropalmatine, magnoflorine, isocorydine and glaucine were prepared separately in methanol (1000 µg/mL). Then, methanol stock solutions containing the eight analytes were prepared and diluted in appropriate concentration to yield a series of concentrations, within the ranges from 0.5 to 1000 ng/mL. The calibration curves were constructed by plotting the value of peak areas versus the value of concentrations of each analyte. All stock solutions were stored in the refrigerator at -20°C until use.

2.3 Instrumentation

Quantitative analysis was performed on a 4000 QTRAP™ MS/MS system, hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystem; Concord, ON, Canada), hyphenated with a Waters ACQUITY UPLC™ system (Waters; Milford, MA, USA) *via* an electrospray (Turbo V™ source with TurboIonSpray™ probe and APCI probe) interface. Waters ACQUITY UPLC™ system was equipped with binary solvent manager, sample manager, column compartment and photodiode array detector (PAD).

2.4 Chromatographic conditions

Chromatographic separation of compounds was obtained with an ACQUITY UPLC CSH™ C18 column (1.7 µm, 2.1 × 100 mm) operated at 25°C.The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and acetonitrile (B), was delivered at a flow rate of 0.3 mL/min under a gradient program: 5% (B) initial to 1.0 min, 5-20% (B) from 1.0 min to 2.0 min, 20-30% (B) from 2.0 min to 3.0 min, 30-90% (B) from 3.0 min to 4.0 min, maintained at 90% (B) from 4.0 min to 5.0 min and back to initial condition from 5.0 min to 5.5 min. The sample injection volume used was 1 µL.

2.5 Mass spectrometric conditions

Positive pneumatically assisted ESI was used for sample introduction, ionization process and operated in the multiple reactions monitoring (MRM) mode. A Turboionspray® probe was vertically positioned 11 mm from the orifice and charged with 5500 V. Source dependent parameters such as temperature (TEM), GS1, GS2 and curtain (CUR) gas were set at 550°C, 50 psi, 50 psi and 20 psi, respectively. The collision-activated dissociation (CAD) gas was set as medium and the interface heater was on. High-purity nitrogen was used for all the processes. The compound-dependent parameters are shown in Table 1. Quadrupole 1 and quadrupole 2 were maintained at unit resolution. AB Sciex Analyst software version 1.5.1 was used to control the LC-MS/MS system and for data acquisition and processing.

Table 1: Optimized compound-dependent parameters by ESI-MS/MS in MRM mode

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	EP (V)	CE (V)	CXP (V)
Magnoflorine (1)	342	297	50	10	27	20
Isocorydine (2)	342	279	73	4.5	27	12
Glaucine (3)	356	325	101	8	20	16
Jatrorrhizine (4)	338	307	50	10	55	15
Tetrahydropalmatine (5)	356	192	86	7	35	7
Tetrahydroberberine (6)	340	176	55	10	35	9
Palmatine (7)	352	336	32	10	40	15
Berberine (8)	336	320	40	10	45	5

3. Results and discussion

3.1 Optimization of chromatographic condition

With respect to UPLC separation, different mobile phases (water-acetonitrile, 0.1% formic acid in water-acetonitrile, water-methanol, 0.1% formic acid in water-methanol) at variable flow rates (0.2, 0.3, and 0.4 mL/min) were examined and compared for better chromatographic separation and appropriate ionization. A mobile phase consisting of 0.1% aqueous formic acid and acetonitrile at a flow rate of 0.3 mL/min resulted in satisfactory separation in a short analysis time. MRM extracted ion chromatogram of analytes are shown in Fig. 2.

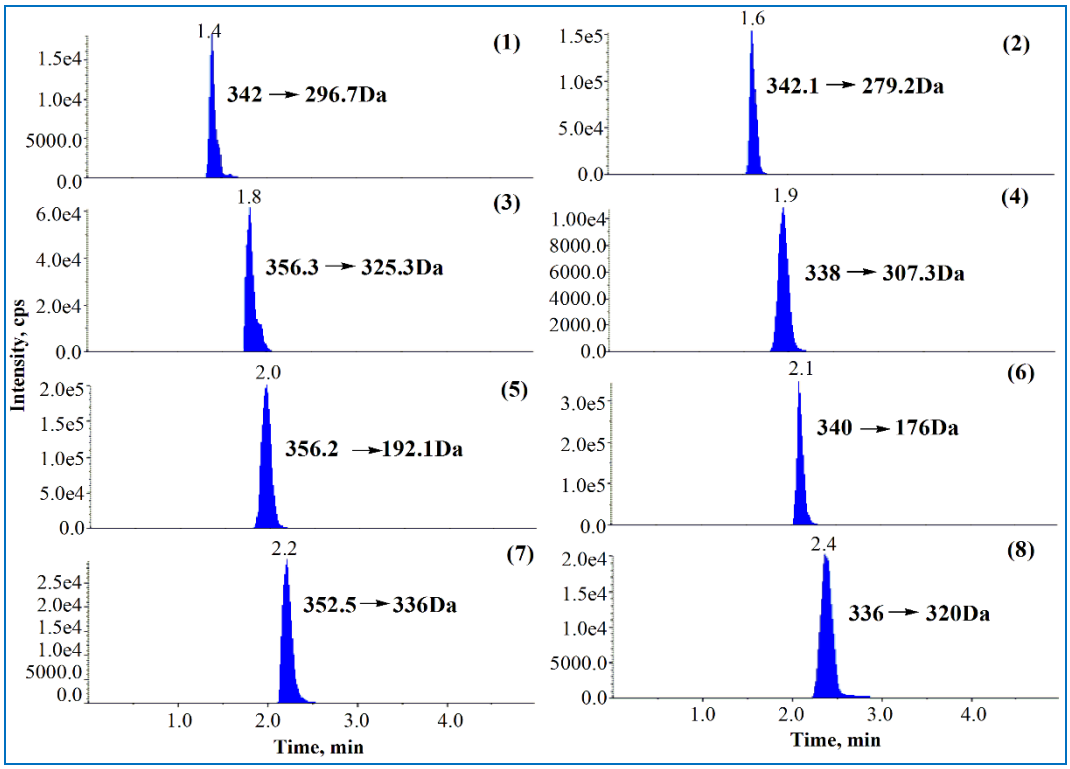

Fig 2: Extracted ion chromatograms (XICs) of analytes in MRM mode

3.2 Optimization of mass spectrometric condition

For quantification of protoberberine and aporphine alkaloids in the *C. fenestratum*, standards magnoflorine (1), isocorydine (2), glaucine (3), jatrorrhizine (4), tetrahydropalmatine (5), tetrahydroberberine (6), palmatine (7) and berberine (8) were studied [2, 7]. Preliminary, compounds dependent MRM parameters (DP: declustering potential; EP: entrance potential; CE: collision energy; and CXP: cell exit potential) were optimized to achieve the most specific and stable MRM transition (precursor-to-product ions). To accomplish this target, each selected analyte (10 ng/mL) was directly injected into the ESI source of mass spectrometer by continuous infusion. The optimized mass spectrometric conditions for the detection of analytes were achieved in positive-ion mode. Analytes 1, 4, 7 and 8 showed $[M]^+$ ion while analytes 2, 3, 5 and 6 showed $[M+H]^+$ ion in Q1 MS scan and, therefore, selected as the precursor ions for MS/MS fragmentation analysis of analytes (Fig. 3). DP and EP were optimized to obtain the maximum sensitivity of $[M+H]^+$ and $[M]^+$ ions in Q1 multiple ion scan (Q1 MI). Identification of the fragment ions and selection of CE for each analyte were carried out in the product ion scan.

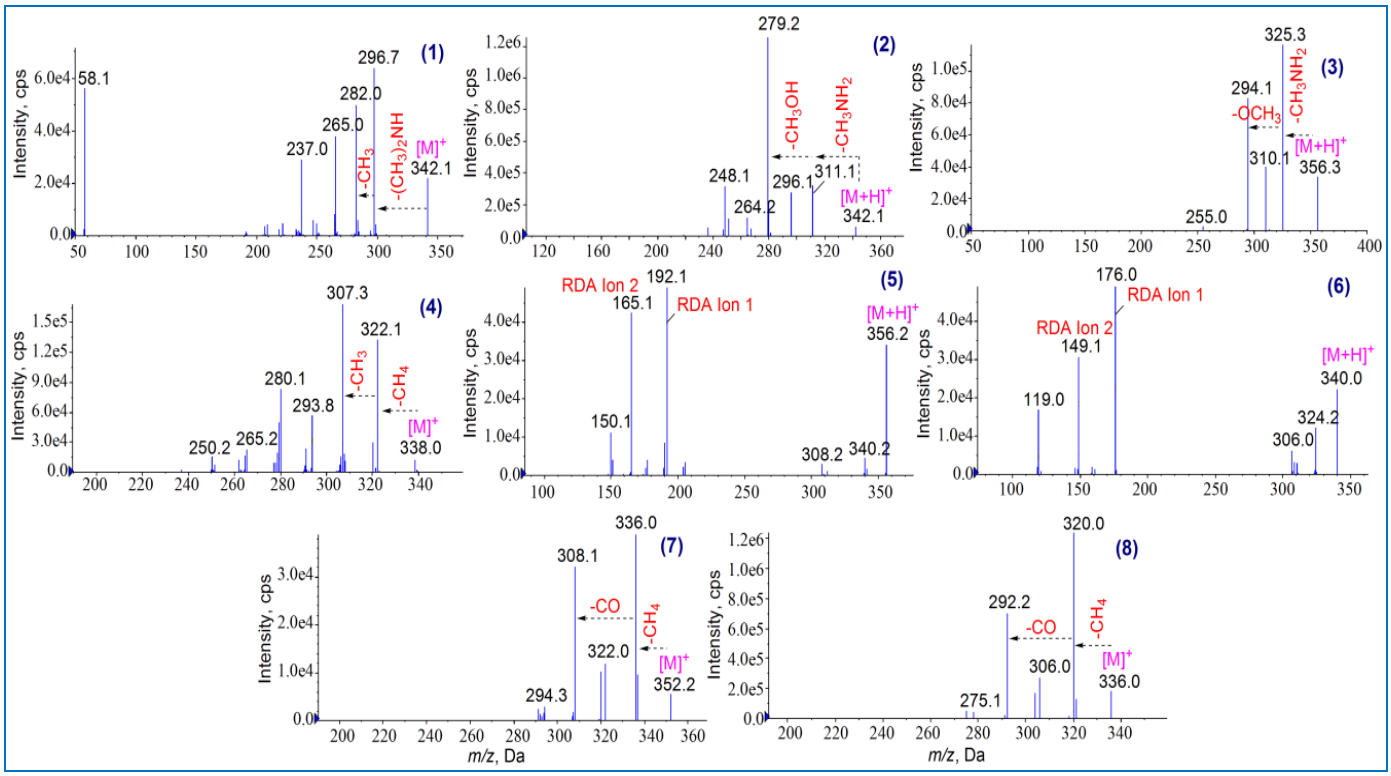

Fig 3: (+)-ESI-MS/MS spectra of selected analytes

Magnoflorine (1) and glaucine (3) showed the most abundant ion at *m/z* 297 and 325 by loss of dimethylamine [$(CH_3)_2NH$] and methylamine [CH_3NH_2], respectively, while isocorydine (2) showed the prominent ion at *m/z* 279 by consecutive loss of methylamine and methanol from the $[M+H]^+$ ion. Similarly, $[M]^+$ ion of jatrorrhizine (4) showed the most abundant ion at *m/z* 307 by consecutive loss of CH_4 and CH_3 while palmatine (7) and berberine (8) generated the prominent ions at *m/z* 336 and 320, respectively, by loss of CH_4. Tetrahydropalmatine (5) and tetrahydroberberine (6) yielded the base peak ion at *m/z* 192 and 176, respectively, by RDA cleavage from the $[M+H]^+$ ion [17]. They were found to be the most abundant, stable and reliable product ions for the analysis. Therefore, they were selected as MRM ion pair (precursor-to-product). Furthermore, CE and CXP were optimized for selected precursor-to-product ions to acquire the maximum sensitivity in MRM scan. The most sensitive and stable transition was selected for the quantification. Table 1 shows the optimized parameters for all the analytes. The source dependent parameters (curtain gas, GS1, GS2 and ion source temperature) were optimized for the highest abundance of precursor-to-product ions by operating UPLC.

3.3 Analytical Method Validation

The proposed UPLC-MRM method for quantitative analysis was validated according to the guidelines of international conference on harmonization (ICH,Q2R1) by determining specificity, linearity, lower limit of detection (LOD), lower limit of quantification (LOQ), precision, solution stability and recovery[18].

3.4 Specificity

Exact identification of each analytes in all the samples is a prerequisite for a successful quantitation. For structural identification, UPLC-ESI-MS/MS method was employed to analyze MRM signals in the sample matrix. All of the peaks of the target analytes in different plant parts of *C. fenestratum* were unambiguously identified by comparison of retention time and MRM transition of standard analytes.

3.5 Linearity, limits of detection (LODs) and quantification (LOQs)

Linearity, LODs and LOQs of eight analytes were determined by serial dilution of sample solutions using the described UPLC-ESI-MS/MS method. The linearity of calibration was performed by the analyte peak area (y) versus concentration (x) and constructed with a weight ($1/x^2$) factor by least-squares linear regression. Good linear relationship between the peak area and concentration was obtained for each of the eight analytes over the tested concentration range with a correlation coefficient (R^2) of ≥0.9988. LODs and LOQs were the concentrations of the compound at which signal-to-noise ratios (S/N) were detected as 3:1 and 10:1, respectively (Table 2). The LOD for each analyte varied from 0.08-0.85 ng/mL and LOQ from 0.26-2.58 ng/mL.

3.6 Precision, Stability and Accuracy

Precision of the developed method was determined at three different levels i.e. intraday variation, interday variation and reproducibility, according to ICH guideline [19]. Intra- and inter-day variations of the method were determine by analyzing known concentrations of the nine analytes in the six replicates during a single day and by triplicating the experiments on five successive days, respectively. Relative standard deviation (RSD) values for precision were in the range of 0.40-2.10% for intraday assays, 0.74-2.37% for interday assays (Table 2). Six different sample solutions of *C. fenestratum* root in parallel were extracted and analyzed with the proposed method to evaluate the reproducibility. The RSD values of eight compounds were in the range of 0.69-3.1%, which showed high reproducibility of method.

Table 2: Linearity, LOD, LOQ, precisions, stability and recovery results of investigated components

Analytes	Linearity			LOD (ng/ml)	LOQ (ng/ml)	Precision RSD(%)			Stability	Recovery	
	Regression equation	R^2	Linear range (ng/ml)			Intraday (n=6)	Interday (n=5)	Reproducibility (n=5)		Mean (n=6)	RSD (%)
1	y=743*x-43.7	0.9999	1.0-250	0.22	0.67	1.49	2.25	1.33	2.01	99.8	0.99
2	y=6047*x-1335	1.0000	2.0-1000	0.73	2.2	1.40	1.61	1.10	1.69	100.3	2.3
3	y=3690*x-49.8	0.9990	0.5-1000	0.19	0.58	0.65	0.74	0.69	1.16	101	3.17
4	y=1100*x-7.06	0.9988	2.0-1000	0.85	2.58	1.49	1.40	1.33	1.92	100.5	1.2
5	y=14600*x-448	0.9998	0.5-200	0.08	0.26	2.10	2.37	0.75	2.54	99.5	2.8
6	y=5094*x+3491	0.9999	2.0-500	0.26	0.79	1.93	1.83	1.71	1.07	98.8	1.1
7	y=989*x+870	0.9992	0.5-50	0.12	0.38	0.40	0.89	3.1	1.15	98	2.6
8	y=1680*x-258	1.0000	1.0-500	0.18	0.55	1.61	1.58	0.91	1.24	98.9	0.59

y: peak area; x: concentration of compound (ng/ml); LOD: limit of detection: S/N=3; LOQ: limit of quantification: S/N=10

Stability of sample solutions stored at room temperature was investigated by replicate injections of the sample solution at 0, 2, 4, 8, 12 and 24 h. The RSD values of stability of the eight analytes were ≤ 2.54% (Table 2). To evaluate the accuracy of this method, a recovery test was applied by the standard addition method. The mixed standard solutions with three different spike levels (low, middle and high) were added into a sample and analyzed using the above mentioned method in triplicate experiments. The recovery was calculated by the formula: recovery = (a-b)/c×100%, where 'a' is the detected amount, 'b' is the original amount and 'c' is the spiked amount. The results showed that the developed analytical method was simple, reliable and reproducible with good recovery in the range of 98-101% (RSD ≤ 3.17%) for all analytes (Table 2).

3.7 Method application

UPLC-ESI-MS/MS method was subsequently applied to determine all the eight bioactive compounds in the ethanolic extracts of *C. fenestratum*. The content of each analyte was calculated from the corresponding calibration curve and summarized in Table 3. The results showed that there were remarkable differences in their contents in all the parts of *C. fenestratum*. Five PBAs were quantified in different plant parts of *C. fenestratum*. In PBAs, the contents of quaternary PBAs (Berberine, Jatrorrhizine and Palmatine) were detected in all plant parts except palmatine in flower which was observed below the detection limit. Berberine was found the most abundant in root part (186.7 mg/g) followed by stem (173.9 mg/g). Contents of jatrorrhizine and palmatine were detected maximum in stem part of the plant (12.75 and 5.24

mg/g, respectively). Tetrahydroprotoberberine alkaloids were detected as minor constituents in each part of plant. Tetrahydropalmatine and tetrahydroberberine were found abundant in stem (0.84 mg/g) and flower (0.007 mg/g), respectively, as shown in Table 3. Content of magnoflorine, isocorydine and glaucine were abundant in leaf (3.80 mg/g), flower (0.76 mg/g) and stem (0.16 mg/g), respectively. Total content of all the eight bioactive compounds were found maximum in root part (200.27 mg/g) followed by stem (194.31 mg/g), leaf (6.31 mg/g) and flower (3.17 mg/g).

Table 3: [a]Content (in mg/g) of eight bioactive compounds in the different parts of *Coscinium fenestratum*

Analytes	Flower	Leaf	Root	Stem
Magnoflorine	0.40±0.021	3.8±0.020	1.05±0.017	0.98±0.011
Isocorydine	0.76±0.013	0.61±0.019	0.36±0.023	0.44±0.020
Glaucine	0.12±0.010	0.11±0.031	0.13±0.023	0.16±0.031
Jatrorrhizine	0.17±0.023	0.39±0.014	9.23±0.031	12.75±0.025
Tetrahydropalmatine	0.47±0.025	0.07±0.018	0.37±0.010	0.84±0.016
Tetrahydroberberine	0.007±0.014	0.003±0.017	0.006±0.021	0.0023±0.017
Palmatine	bdl	0.00078±0.037	2.42±0.017	5.24±0.002
Berberine	1.25±0.030	1.33±0.017	186.7±0.008	173.9±0.001
Total	3.17	6.31	200.27	194.31

bdl: below detection level; [a]Content=mean±SD (n=3); [b]Total: The total contents of eight compounds in *C. fenestratum* extract

4. Conclusions

In summary, the present study involved quantification of eight bioactive compounds (protoberberine and aporphine alkaloids) in different plant parts of *C. fenestratum*. An UPLC-ESI-MS/MS method under MRM mode was successfully developed and validated as per ICH guidelines and applied in samples. Results indicate that total content of 8 bioactive compounds are found to be maximum in the root part. Berberine was found the most abundant compound in root part followed by stem. Results also showed that UPLC-ESI-MS/MS with MRM acquisition is more suitable method for multi-components analysis. This method has highly sensitive and precise which is able to demonstrate the quality of the herbal products from different manufacturers.

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