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Determination of bioactive isoquinoline alkaloids in *Thalictrum reniforme* Wallich and *Thalictrum neurocarpum* Royale using ultra performance liquid chromatography with hybrid triple quadrupole linear ion trap mass spectrometer

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Abstract
Thalictrum is a well recognised genus of medicinal plants. It is widely distributed in temperate and tropical habitats of India. Alkaloid isomers from Thalictrum are reported to exhibit various pharmacological activities like antiamebic, antimicrobial, antitumour and antiviral. *Thalictrum reniforme* Wallich and *Thalictrum neurocarpum* Royale are very less studied species of the genera. In present study, we have discussed about simultaneous quantification of seven bioactive alkaloids in aerial parts of *T. reniforme* and *T. neurocarpum* using ultra performance liquid chromatography attached with hybrid triple quadrupole linear ion trap mass spectrometer (UPLC-QqQ_{LIT}-MS) operated in the multiple reactions monitoring (MRM) acquisition. The chromatographic separation of all the standards were carried out by using ACQUITY UPLC CSH™ C_{18} column using a gradient programming at a flow rate of 0.3 mL/min. Method was validated by determining the precision and recovery values of the samples in terms of relative standard deviations (RSDs) which was found 0.40-3.1% and 0.59-3.17% (98-101%), respectively. Calibration curves for all the seven analytes was determined as optimum linear detector response having $R^2 \geq 0.9988$ over the concentration range of 0.5-1000 ng/mL. The validated method was then used to quantify the isoquinoline alkaloids which revealed that *T. reniforme* had high level of total content as compared to *T. neurocarpum*. The quantity of analyte was subjected to principal component analysis.

Keywords: Alkaloids, Quantification, Thalictrum, UPLC-ESI-QqQ_{LIT}-MS/MS

1. Introduction
Thalictrum (family Ranunculaceae) is very widely distributed genus of medicinal plants in China, India, Japan, Russia and found in temperate and tropical habitats [1, 2]. The phytochemistry of this genus has attracted much attention and till date more than 250 alkaloids have been reported from approximately 100 species of the genus [3, 4]. Several Thalictrum species are used in folk medicine for the treatment of jaundice, rheumatism and are useful as diuretic, stomachic, antiseptic, aperient etc., [5, 6]. The alkaloid isomers isolated from many Thalictrum species are also reported to exhibit various pharmacological activities like antiamebic, antimicrobial, antitumour and antiviral [7-9]. These natural products are pharmacologically and structurally important as they exhibit specific antiparasitic properties, such as antileishmanial and antimalarial and are characteristic for many species [2, 10-12]. Alkaloids are well known pharmacologically active substances of Thalictrum genus but isoquinoline alkaloids are the most explored [13-14]. Several bisbenzylisoquinolines are also isolated from aerial parts and roots of many Thalictrum species [15]. The accumulation of quaternary isoquinoline alkaloids such as berberine and magnofluorine are also reported for the several pharmacological activities [16]. Isoquinoline alkaloids are characteristic metabolites which may be considered as significant chemotaxonomic markers for the genus [17]. Thalictrum reniforme Wallich and Thalictrum neurocarpum Royale are very less studied and similar looking species of the genera. Difference in many pharmacological activities of the herbs usually related with differences in chemical composition and thus affecting the commercial value of their herbal products.

Therefore the identification and quantitation of alkaloids is important for differentiation of these two morphologically similar medicinal herbs.

Several techniques have been used for the determination of isoquinoline alkaloids in plant extract [18]. These methods are based on gas chromatography (GC), capillary electrophoresis (CE), direct analysis in real time mass spectrometry (DART MS), high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and high performance TLC (HPTLC) [18-22]. The UPLC-ESI-MS/MS method in multiple reactions monitoring (MRM) acquisition for determination of multicomponent has proven to be more powerful analytical technique in comparison with earlier applied analytical methods [23]. It has rapid separation power combined with multiple ion detection based on selective ion fragmentation which is important for absolute quantitation of many compounds simultaneously in a crude extract [24]. This UPLC-ESI-MS/MS approach is more reliable and applicable to quality control of herbal/polyherbal preparations and useful for pharmaceutical research [25-26].

2. Materials and methods

2.1 Materials

Plant material was collected from the Churdhar, Himachal Pradesh and voucher specimen of *T. reniforme* (V S. No. 9738) and *T. neurocarpum* (V S. No. 2299) was deposited in the Department of Forest Products, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India.

AR grade ethanol, purchased from Merck Millipore (Darmstadt, Germany), was used in the preparation of ethanolic extract of aerial part of plants. LC-MS grade methanol, acetonitrile and formic acid, purchased from Sigma-Aldrich (St. Louis, MO, USA), were used in mobile phase and sample preparation during the LC-MS studies. Ultra-pure water was obtained from Direct-Q system (Millipore, Billerica, MA, USA). Standard compounds of berberine, palmatine, jatrorrhizine, magnoflorine, tetrahydroberberine, tetrahydropalmatine, glaucine and isocorydine (Figure. 1) were purchased from Shanghai Tauto Biotech Co., Ltd (Shanghai, China).

2.2 Extraction and Sample Preparation

The shade dried aerial part of plants was grinded into fine powder using mechanical mill. 100 g powders of each plant were suspended with 500 mL ethanol. The suspensions was initially sonicated for 30 min in ultrasonic water bath (53 KHz) at 30˚C and then allowed to stand at room temperature for twenty four hours. 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 and temperature at 20-50 kPa and 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).

2.3 Preparation of standard solutions

Stock solutions of reference standards containing berberine, palmatine, jatrorrhizine, tetrahydroberberine, tetrahydropalmatine, magnoflorine, isocorydine and glaucine were prepared separately in methanol (1000 µg/mL). Stock solutions containing the seven analytes were further 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 analytes. All stock solutions were stored at -20°C.

2.4 Instrumentation

A 4000 QTRAP™ MS/MS system consist of hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystem; Concord, ON, Canada), hyphenated with Waters ACQUITY UPLC™ system (Waters; Milford, MA, USA) *via* a pneumatically assisted electrospray (Turbo V™ source with Turbo Ion Spray™ probe) interface was used for quantitative analysis. Waters ACQUITY UPLC™ system was equipped with binary solvent manager, sample manager, column compartment and photo diode array detector (PDA).

2.5 Chromatographic conditions

ACQUITY UPLC CSH™ C_{18} column (1.7 µm, 2.1 × 100 mm) operated at 25°C was used for separating the compounds. Mobile phase, consisted of 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.

2.6 Mass spectrometric conditions

ESI in positive mode was used for ionization process and operated in the multiple reactions monitoring (MRM) mode. A Turboionspray® probe was positioned vertically at 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 Supporting Information Table S1. Quadrupole 1 and quadrupole 2 were maintained at unit resolution. AB Sciex Analyst software version 1.5.1 was used for data acquisition and processing.

2.7 Statistical analysis

All statistical analysis was carried out using Minitab 14 (trial Version). All the statistical calculations related to quantitative analysis were performed using Graph Pad Prism software version 5. Data for statistical analysis was prepared from content of three repeats of each sample and summarized as Mean ± SD (standard deviation) Analyses were performed on standard statistical software STATISTICA windows version 7.0 (Tulsa, Oklahoma, USA).

3. Results and discussion

3.1 Optimization of chromatographic condition

To achieve 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 during UPLC method development. A mobile phase consisting of 0.1% aqueous formic acid and acetonitrile at a flow rate of 0.3 mL/min was finally selected for chromatographic separation in a short analysis time. MRM

extracted ion chromatogram of analytes are shown in Fig. 2.

3.2 Optimization of mass spectrometric condition

Standard compounds of magnoflorine (1), isocorydine (2), glaucine (3), jatrorrhizine (4), tetrahydropalmatine (5), tetrahydroberberine (6), palmatine (7) and berberine (8) were used to optimize the mass spectrometric conditions. The MS/MS spectrums of all seven analytes were shown in Figure. 2. Compounds dependent MRM parameters such as DP (declustering potential), EP (entrance potential), CE (collision energy), and CXP (cell exit potential) were optimized to get the most specific and stable MRM transition (precursor-to-product ions). Each analytes with concentration 10 ng/mL was injected into the positive ion ESI source using continuous infusion. Precursor ions of analytes were identified in Q1 MS scan and selection of CE, and product ions were obtained in the product ion scan. DP and EP for precursor ion were optimized in Q1 multiple ion scan (Q1 MI). MRM scan was then used to optimize CE and CXP for selected precursor-to-product ions. Other source dependent parameters such as curtain gas, GS1, GS2 and ion source temperature were optimized for the intense peak of precursor-to-product ions in the flow injection analysis (FIA).

3.3 Analytical Method Validation

The developed UPLC-MS method 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, stability and recovery [27].

3.3.1 Specificity

Two MRM signals as quantifier and qualifier were used in the method for exact identification of each analytes in the samples. All analytes in aerial part of *T. reniforme* and *T. neurocarpum* were unambiguously identified by comparison of retention time, quantifier and qualifier transitions (Table S1). Extracted ion chromatograms (XIC) of seven bioactive compounds identified from *T. reniforme and T. neurocarpum* were shown in Figure 3.

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

The standard stock solution of seven analytes was diluted to different working concentrations of 0.5, 1, 10, 25, 50, 75, 100, 250, 500, 1000 ng/mL for the construction of calibration curves and detection of linearity, limits of detection (LODs) and limits of quantification (LOQs). The linearity of calibration was obtained by the analytes peak area (y) versus nominal concentration (x) and constructed with a weight ($1/x^2$) factor by least-squares linear regression. The linearity calibration curves were made on the basis of five experiments of each reference compound. Optimum linear relationship was obtained for each of the seven analytes over the tested concentration range with a correlation coefficient value (R^2) of ≥0.9988. The LOD for each analytes varied from 0.19-2.92 ng/mL and LOQ from 0.26-8.79 ng/mL (Table 1).

3.3.3 Precision, Stability and Accuracy

On analyzing known concentrations of the seven analytes in the six replicates during a single day and by triplicating the experiments in three successive days respectively, intra- and inter-day variations of the method were determined. Relative standard deviation (RSD) values for precision were in the range of 0.40-2.10% for intra-day assays, and 0.74-2.37% for inter-day assays (Table 1). Six different sample solutions of *T. reniforme* and *T. neurocarpum* aerial part were analyzed to determine the reproducibility of the developed method. The RSD values of seven compounds were in the range of 0.69-3.1%, which showed high reproducibility of method.

Stability of sample were determine by replicate injections of the sample solution at 0, 2, 4, 8, 12 and 24 h and RSDs value for stability were found in range of 0.79–2.12% (Table 1). Accuracy of the method was detected by the recovery of analytes using recovery test by adding a standard solutions into a sample with three different spike levels at 50%, 100% and 200% of each reference standard. The detected recovery from triplicate experiments was found in range of 98-101% (RSD ≤ 3.17%) for all analytes (Table 1). The results showed that the developed analytical method was simple, reliable and reproducible with accepted limit of recovery.

3.4 Method application

The developed and validated UPLC-ESI-MS/MS method was successfully utilized to determine all the seven bioactive compounds in the ethanolic extracts of *T. reniforme* and *T. neurocarpum* aerial part. The results calculated from the corresponding calibration curve revealed differences in the contents of all analytes from *T. reniforme* to *T. neurocarpum* aerial part as shown in Table 2. The contents of quaternary proto berberine alkaloids (berberine and jatrorrhizine) were detected in both plants. Berberine content was found almost similar in *T. reniforme* (5.71 mg/g) and *T. neurocarpum* (5.82 mg/g) while the jatrorrhizine content was abundant in *T. reniforme* than *T. neurocarpum* (2.03 and 0.54 mg/g, respectively). Tetrahydropalmatine was found slight abundant in *T. neurocarpum* (0.30 mg/g) and as shown in Table 2. Content of magnoflorine, isocorydine and glaucine were most abundant in *T. reniforme* (83.74 mg/g, 29.13 mg/g and 17.19 mg/g, respectively). Total content of all the seven bioactive compounds were found maximum in *T. reniforme* (138.11 mg/g) followed by *T. neurocarpum* (194.31 mg/g). Chlorogenic acid was also detected along with these alkaloids and found approximately same in both *T. reniforme* and *T. neurocarpum* aerial part (0.76 mg/g and 0.16 mg/g respectively).

3.5 Comparison between *T. reniforme and T. neurocarpum*

Estimation of isoquinoline alkaloids supports the chemotaxonomic evaluation and useful for differentiation of plants among the genus [27]. In both *T. reniforme* and *T. neurocarpum* all the identified isoquinoline alkaloids and chlorogenic acid were scrutinized for their influence as a possible chemotaxonomy marker. As the multivariate data can be displayed in the form of a two-dimensional graph of three or more quantitative variables starting from the same point using radar chart, the quantitative variation of all the identified isoquinoline alkaloids in both species is represented graphically as shown in Figure 4. It is evident from the result that in both species there is difference in alkaloid content and isoquinoline alkaloids such as aporphine (magnoflorine, isocorydine and glycine) alkaloid can be used as chemotaxonomic marker to differentiate the two medicinally active species.

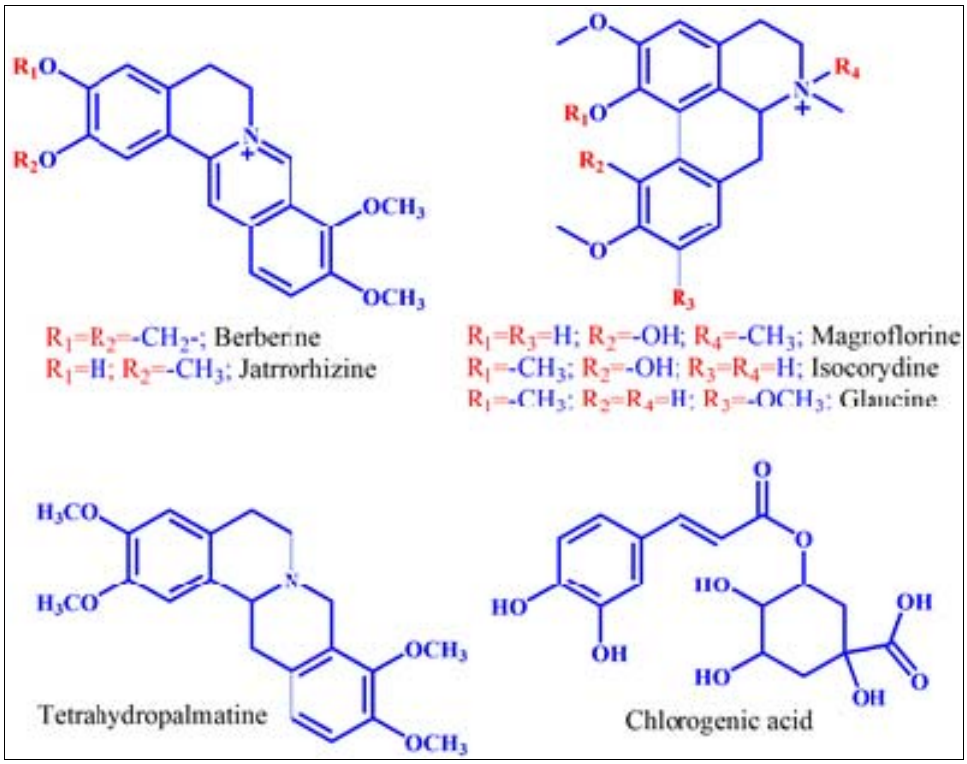

Fig 1: Structures of seven bioactive compounds from *T. reniforme and T. neurocarpum*

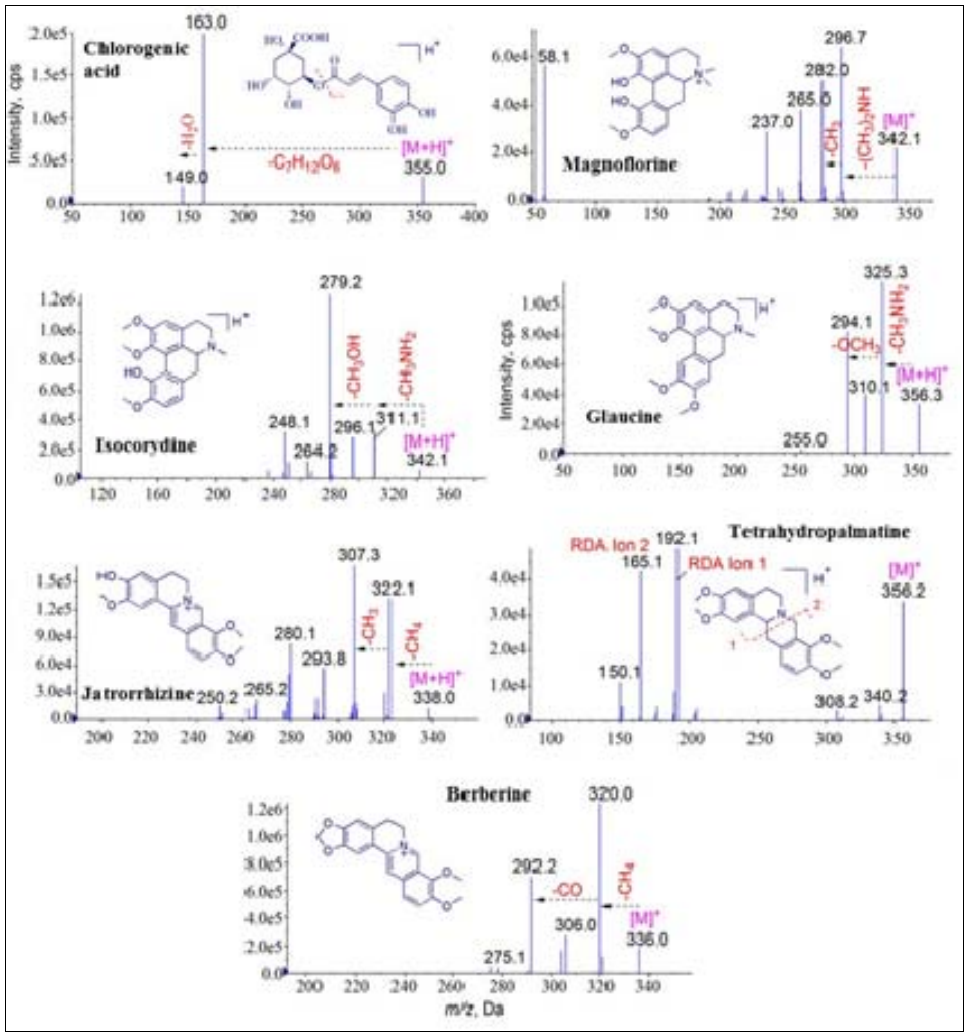

Fig 2: MS/MS spectrum of analytes by ESI-MS/MS

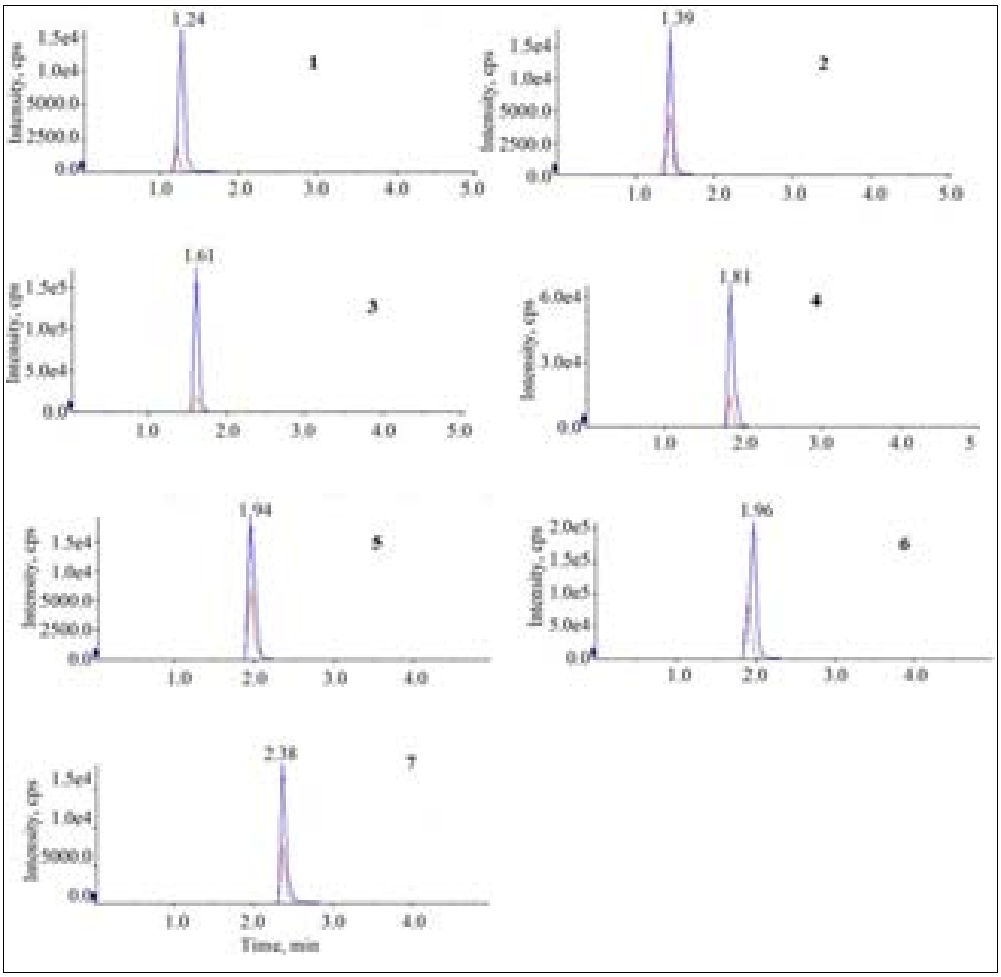

Fig 3: Extracted ion chromatograms (XICs) of analytes in MRM mode

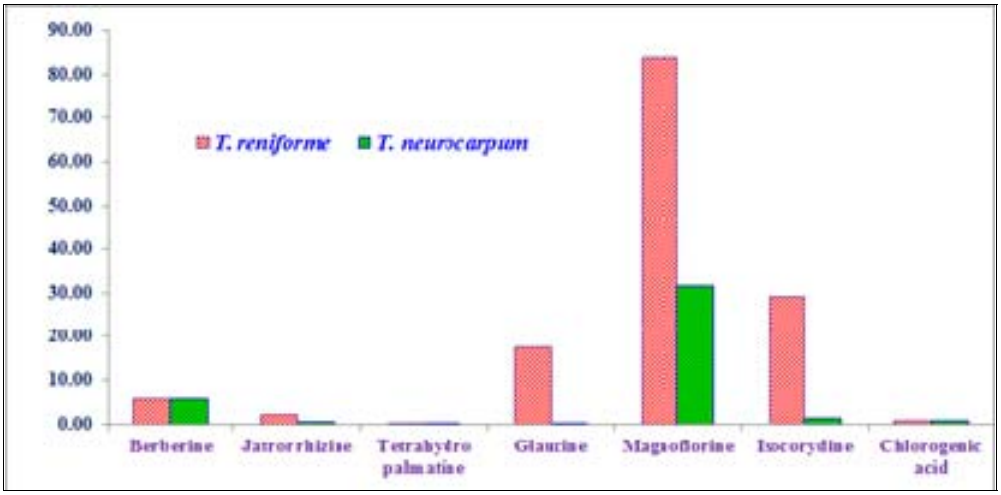

Fig 4: Comparative difference in quantity of all analytes in *reniforme and T. neurocarpum*

Table 1: Compound dependent parameters (MRM) of analytes

Peak No.	Analytes	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (ms)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
1.	Chlorogenic Acid	1.24	355.3	163.1	200	50	12	15	12
2.	Magnoflorine	1.39	342.0	296.7	200	50	10	27	20
3.	Isocorydine	1.61	342.1	279.2	200	73	4.5	27	12
4.	Glaucine	1.81	356.3	325.3	200	101	8	20	16
5.	Jatrorrhizine	1.94	338.0	307.3	200	50	10	55	15
6.	Tetrahydropalmatine	1.96	356.2	192.1	200	86	7	35	7
7.	Berberine	2.38	336.0	320.0	200	40	10	45	5

DP-declustering potential; EP-entrance potential; CE-collision energy; CXP-cell exit potential

Table 2: Linearity, precision, stability and recovery for selected analytes

Peak No.	Analytes	Linearity					Precision (%RSD)			Stability %RSD (n=6)	Recovery (n=6)	
		Linear range (ng/mL)	R^2	Regression equation	LOD (ng)	LOQ (ng)	Intraday (n=6)	Interday (n=6)	Reproducibility (n=6)		Mean	RSD (%)
1.	Chlorogenic acid	0.5-1000	1.0000	y= 610*x-19.7	0.12	0.35	2.40	2.39	1.07	2.82	91.20	1.20
2.	Magnoflorine	0.5-250	0.9999	y= 743*x-43.7	0.22	0.67	1.42	2.25	0.41	2.64	97.49	2.30
3.	Isocorydine	0.5-1000	0.9991	y= 6060*x-266	0.36	1.08	1.48	1.65	1.11	1.98	99.60	0.92
4.	Glaucine	0.5-1000	0.9990	y= 3690*x-49.8	0.19	0.56	0.65	0.74	0.69	2.21	101.50	1.49
5.	Jatrorrhizine	0.5-1000	0.9988	y= 1100*x-7.66	0.87	2.65	1.53	0.96	1.02	2.02	99.01	1.32
6.	Tetrahydropalmatine	0.5-200	0.9998	y= 14600*x-448	0.08	0.26	2.10	2.37	0.75	3.27	102.10	1.60
7.	Berberine	1-100	0.9989	y = 1030*x-487	1.53	4.64	1.52	1.68	0.75	1.72	98.60	1.11

Table 3: Contents (mg/g) of analytes in aerial part of *T. reniforme and T. neurocarpum* (n=6)

Peak No.	Analytes	*T. reniforme*	*T. neurocarpum*
1.	Chlorogenic acid	0.78	0.71
2.	Magnoflorine	83.74	31.43
3.	Isocorydine	29.13	1.37
4.	Glaucine	17.29	0.14
5.	Jatrorrhizine	2.03	0.54
6.	Tetrahydropalmatine	0.22	0.30
7.	Berberine	5.71	5.82
	Average Content of all analytes	138.9	40.31

Table 4: Supporting Information's: Supplementary Table S1. UPLC gradient condition

Time (min.)	% A (.1% (v/v) formic acid aqueous solution)	% B (acetonitrile)	Flow rate (mL/min)
Initial	95.00	5.00	0.300
1.00	95.00	5.00	0.300
2.00	80.00	20.00	0.300
3.00	70.00	30.00	0.300
4.00	10.00	90.00	0.300
5.00	10.00	90.00	0.300
6.00	95.00	5.00	0.300

Column; ACQUITY UPLC CSH^{TM} C_{18} column (1.7 µm, 2.1×100 mm).
Injection volume; 1µl, Column temperature; 25°C

Table 5: Supplementary Table S2. Compounds identified in Tinospora cordifolia stem

Peak No.	Calculated *m/z*	Peak type	Formula	Fragments *m/z* (abundance)	Identification
1.	355.0	$[M+H]^+$	$C_{16}H_{18}O_9$	355.0 (18), 163.0 (100), 149.0 (14)	Chlorogenic acid
2.	342.1	$[M]^+$	$C_{20}H_{24}NO_4^+$	342.1 (29), 296.7 (100), 282.0 (71), 265.0 (54), 237.0 (38)	Magnoflorine
3.	342.1	$[M+H]^+$	$C_{20}H_{23}NO_4$	342.1 (7), 311.1 (24), 296.1 (19), 279.2 (100), 264.2 (14), 248.1 (28)	Isocorydine
4.	356.3	$[M+H]^+$	$C_{21}H_{25}NO_4$	356.3 (32), 325.3 (100), 310.1 (35), 294.1 (63), 255.0 (3)	Glaucine
5.	338.0	$[M]^+$	$C_{20}H_{20}NO_4^+$	338.0 (5), 322.1 (67), 307.3 (100) 293.8 (33), 280.1 (42), 265.2 (11) 250.2 (7)	Jatrorrhizine
6.	356.2	$[M+H]^+$	$C_{21}H_{26}NO_4$	356.2 (54), 340.2 (8), 308.2 (5), 192.1 (100), 165.1 (71), 150.1 (21)	Tetrahydro palmatine
7.	336.0	$[M]^+$	$C_{20}H_{24}NO_4^+$	336.0 (18), 320.0 (100), 306.0 (26), 292.2 (58), 275.1 (4)	Berberine

4. Conclusion

The present study involved quantification of seven bioactive compounds in aerial parts of *T. reniforme and T. neurocarpum*. An UPLC-ESI-MS/MS method under MRM mode was successfully applied in samples. Results indicate that total content of seven bioactive compounds are found to be maximum in the *T. reniforme*. Magnoflorine was found the most abundant compound in both plants. 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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