Quantitative Determination of Four Constituents of *Tinospora sps.* by a Reversed-Phase HPLC–UV–DAD Method. Broad-Based Studies Revealing Variation in Content of Four Secondary Metabolites in the Plant from Different Eco-Geographical Regions of India

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Abstract

This paper describes the separation and quantitation of important markers, such as 20β-hydroxyecdysone, tinosporaside, cordioside, and columbin, present in three species of Tinospora viz, *T. cordifolia, T. malabrica, and T. crispa.* A reverse-phase (RP) high-performance liquid chromatography (HPLC)–UV–diode array detection (DAD) method employing gradient elution is thus developed. The marker compounds isolated from 70% ethanolic extract of *T. cordfolia* by repeated column chromatography are identified on the basis of ^{1}H NMR, ^{13}C NMR, and mass spectral data. The compounds are separated on a RP (RP-18, 5 µm, 250 × 4.6-mm i.d.) column using water–acetonitrile gradient and are detected by the HPLC–UV–DAD method. The calibration curves that result from marker compounds in the concentration range of 100–2000 ng on column exhibit a good correlation ($r^2 \geq 0.99978$). The method is successfully applied to separate and study the content of four marker compounds in 40 different accessions of three Tinospora species collected from different regions of India. The studies reveal that the maximum amount of the marker compounds is present in *Tinospora cordifolia* species, especially from accessions collected from higher altitudes of the Jammu province (North India).

Introduction

Tinospora (Manisbermaceae) is a climber distributed throughout tropical and subtropical India (1), and only three species of the plant are found in the Indian subcontinent. The three species include: *T. cordifolia* Miers, *T. malabarica* Miers, and *T. crispa* Miers. *T. cordifolia* is widely distributed throughout all the tropical regions of the country. However, in the northwestern regions, especially Jammu and Himachal Pradesh, it is in abundance. Unlike the wide distribution of *T. cordifolia*, the other two species of the plant (*T. malabarica* and *T. crispa*) are distributed over limited areas. *T. malabarica* is found in the southern Kerala region of the country, and *T. crispa* grows only in northeastern Himalayas of Assam (2). The plant is well known for its adaptogenic, immunomodulatory activity (3–6). The stem of the plant is one of the main constituent of several Ayurvedic/herbal preparations that are used in general debility, dyspepsia, fever, and urinary diseases. It is bitter, stomachic, diuretic, stimulates bile secretion, causes constipation, allays thirst, burning sensation, vomiting, enriches the blood, and cures jaundice (7). The extract of the plant stem is useful in the management of skin disorders (8,9). Dry bark of *T. cordifolia* has anti-spasmodic, antipyretic, anti-allergic, anti-inflammatory, and antileprotic properties (10–14). The roots of the plant are a powerful emetic and are used for visceral obstructions. A galaxy of organic molecules with diverse structures, such as alkaloids, terpenoids, glycosides, sterols, lactones, and fatty acids, have been reported from this herb. Important bioactive principles reported from this herb include tinosporide, tinosporaside, tinosporadine, cordifol, cordioside, columbin, and 20β-hydroxyecdysone.

With the importance of the plant in the traditional and folklore systems of medicine in mind and in continuation of our program on herbal drugs (15,16), it was decided to carry out broad-based studies to identify those species of *Tinospora*, which could express four selected biomarkers (Figures 1–4) in appreciable quantities. To support these types of studies, a sensitive and specific assay technique was needed for the quantitation of amounts of the four marker compounds in different accessions of the plant collected from different locations. These studies were carried out with the high-perfromance liquid chromatography (HPLC) method. The qualitative as well as quantitative HPLC analysis under standard

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conditions provides chromatograms, which are very useful for controlling the quality of phytopharmaceuticals. Although thin-layer chromatography is a powerful and simple technique used for this purpose, there are situations where this technique did not give satisfactory results because of its own limitations (17). HPLC–UV–diode array detection (DAD) is a well-suited analytical tool for these types of studies, and the method was developed to fulfill the objective of the conceived project.

Experimental

Solvents and chemicals

All the solvents used in this study were of HPLC grade. Both HPLC-grade methanol and acetonitrile were purchased from Ranbaxy chemicals (Mohali, Punjab, India) and water was prepared with a Milli-Q water purification system (Millipore, Billerica, MA). All four marker compounds were isolated from a 70% ethanol extract of the *Tinospora cordifolia* plant. The purity of the markers was ≥ 98%, which was established by HPLC.

Plant material

Tinospora cordifolia plant material was collected from Ramnagar (Udhampur District), Jammu, and Kashmir (India) for isolation of the marker compounds, as the plants from this region of the country contained the said marker compounds in sufficient amounts. For broad-based studies, all of the three species (*Tinospora cordifolia*, *Tinospora malabarica,* and *Tinospora crispa*) were collected from different regions of India. The specimen vouchers of all three species of Tinospora were deposited in the herbarium division of the institute (RLL, Jammu, India).

Isolation and characterization of marker compounds

Plant material (3 kg) was extracted with 70% ethanol at room temperature by the percolation method. The water–alcohol (70:30) extract was stripped of ethanol, and the aqueous matrix left behind was successively extracted with chloroform, ethyl acetate, and, finally, with *n*-butanol. The *n*-butanol extract was made free from the solvent under reduced pressure on a rotavapour to give residue (45 g). The residue obtained after solvent evaporation was passed through a 900-g silica gel (60–120 mesh) column. The elution of the *n*-butanol extract residue in chloroform with increasing polarity of methanol yielded four marker compounds: 20β-hydroxyecdysone (Figure 1) (0.2%), tinosporaside (Figure 2) (0.04%), cordioside (Figure 3) (0.15%), and columbin (Figure 4) (0.006%). These compounds were characterized on the basis of their ^{1}H NMR, ^{13}C NMR, ^{13}C DEPT (45° and 135°), and mass spectral data. The data of the isolated compounds was in agreement with the data reported in literature (18,19).

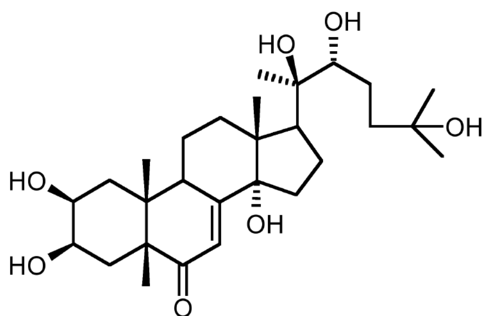

Figure 1. 20β-hydroxyecdysone.

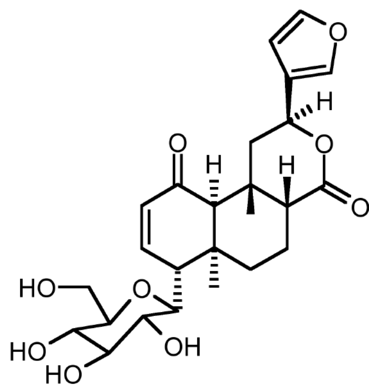

Figure 2. Tinospraside.

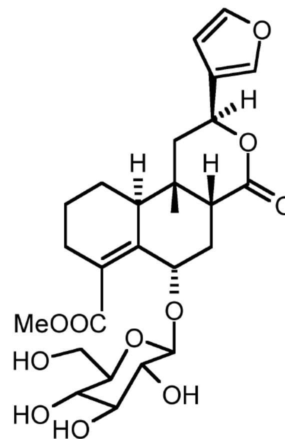

Figure 3. Cordioside.

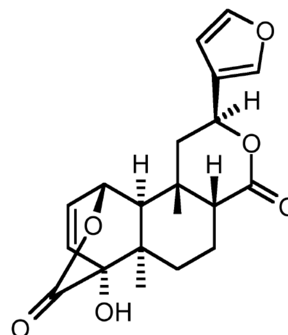

Figure 4. Columbin.

Sample preparation

Dried and powdered leaves (50 mg) were extracted with 70% ethanol thrice at room temperature. All three extracts were combined and evaporated under reduced pressure to give residues in different amounts (Table I). Accurately weighed quantities (100 mg) of residue from each sample were dissolved in 2 mL of an HPLC-grade water–methanol (1:1) mixture. The solutions were filtered through a millipore filter (0.2 µm) before injection onto the HPLC system.

Preparation of the stock solutions and calibration standard solutions

The stock solutions (4 mg/mL) of the marker compounds (Figures 1–4) were prepared in HPLC-grade methanol and stored in a refrigerator at 4°C. From the stock solutions, working solutions of six different concentrations (40, 80, 160, 320, 400, and 800 ng/µL) were prepared by dilution with HPLC-grade methanol. An artificial mixture of the four marker compounds was prepared by mixing the working solutions in equal volumes to give six final concentrations (10, 20, 40, 80, 100, and 200 ng/µL) each of the marker compound in the six different solutions, respectively. Ten microliters of each of these solutions was i njected to give six concentration levels in the range of 100–2000 ng on the column for preparation of calibration curves and HPLC–UV–DAD studies. The analysis was carried out thrice at each level.

Instruments

The marker compounds were separated and quantitated by using a Shimadzu HPLC system (Kyoto, Japan) consisting of an LC-10 ATvp pump, SIL-10 ADvp automatic sampling unit (auto sampler), CTO-10 ASvp column oven SPD-M10 Avp DAD, and SCL-10 Avp as the system controller. Class VP software (version 6.10) (Shimadzu) was used for data analysis and data processing. The samples were analyzed at 30°C on an RP-18, 5 µm, 250 × 4-mm i.d. Merck (Darmstadt, Germany) column. UV–DAD detection was performed at 215 nm. The analysis was carried out using a gradient of water–acetonitrile initially at 90:10, changing to 0:100 in 40 min, followed by isocratic elution at 0:100 for 5 min, changing to 90:10 in 10 min, and,

Table I. Amounts of Marker Compounds (Figures 1–4) in *T. cordifolia sps.*

S. no.	Accession code	Origin	Weights of extracts in grams	20β-hydroxy -ecdysone		Tinosporaside		Cordioside		Columbin	
				Extract (%)	Plant (%)	Extract (%)	Plant (%)	Extract (%)	Plant (%)	Extract (%)	Plant (%)
1	RRLJ (82-12)	Bakshi Nagar*	1.059	6.54	0.130	1.38	0.027	6.120	0.122	6.34	0.126
2	RRLJ (82-14)	Ram Nagar*	1.405	90.49	2.53	1.519	0.042	1.757	0.049	2.014	0.056
3	RRLJ (82-5)	Mandal*	1.289	2.68	0.063	0.750	0.018	0.940	0.022	1.180	0.028
4	RRLJ (82-28)	Joginder Nagar*	1.714	4.753	0.161	0.446	0.015	1.390	0.047	1.935	0.65
5	RRLJ (82-27)	Mandi†	1.000	7.058	0.141	0.887	0.017	1.506	0.030	0.219	0.004
6	RRLJ (82-1)	Dadatalab*	1.506	1.68	0.050	0.654	0.019	0.743	0.022	0.511	0.015
7	RRLJ (82-6)	Nandka*	1.032	5.86	0.117	1.486	0.029	0.639	0.012	0.792	0.015
8	RRLJ (82-11)	Talab Tilo*	1.114	8.91	0.19	0.690	0.15	1.43	0.031	0.84	0.018
9	RRLJ (82-25)	Tandla*	1.03	0.943	0.018	0.299	0.005	0.440	0.008	0.503	0.010
10	RRLJ (JH-6)	Dwarka‡	1.296	0.884	0.021	0.542	0.013	0.433	0.010	0.481	0.011
11	RRLJ (82-8)	Madana*	1.200	25.72	0.617	0.654	0.015	0.296	0.007	0.280	0.006
12	RRLJ (JH-11)	Kalindi Kung‡	1.248	1.150	0.027	0.830	0.019	0.610	0.014	0.705	0.016
13	RRLJ (82-10)	Palampur†	1.302	1.983	0.050	1.320	0.034	0.888	0.023	1.954	0.050
14	RRLJ (82-29)	Chamba†	1.503	1.798	0.053	0.564	0.016	0.798	0.023	0.513	0.015
15	RRLJ (JH-7)	Ridge karolbagh‡	1.500	8.670	0.260	1.532	0.045	0.860	0.025	0.899	0.026
16	RRLJ (JH-2)	Khuraj‡	1.059	6.54	0.130	1.38	0.027	0.612	0.012	0.634	0.012
17	RRLJ (JH-9)	Mehroli‡	1.011	0.47	0.009	0.38	0.007	0.455	0.009	0.492	0.009
18	RRLJ (JH-10)	Munirca‡	1.320	4.73	0.141	0.886	0.026	5.47	0.164	0.772	0.023
19	RRLJ (82-17)	Nandni*	1.288	4.80	0.115	0.716	0.017	0.684	0.016	0.650	0.015
20	RRLJ (82-23)	Akhnoor*	1.265	6.97	0.167	1.232	0.029	0.986	0.023	0.832	0.019
21	RRLJ (82-4)	Khada‡	1.246	2.64	0.063	0.750	0.018	0.940	0.022	1.180	0.028
22	RRLJ (82-22)	Chidi‡	1.224	5.83	0.139	0.849	0.020	0.643	0.015	0.605	0.014
23	RRLJ (82-16)	Nagrota‡	1.258	1.868	0.044	0.543	0.013	0.480	0.011	0.497	0.011
24	RRLJ (Hr-1)	Rohtak§	1.296	2.40	0.057	1.932	0.046	0.860	0.020	0.497	0.011
25	RRLJ (Hr-2)	Karnal§	1.360	4.96	0.128	3.260	0.084	0.986	0.025	1.036	0.026
26	RRLJ (Hr-3)	Hissar§	1.263	5.62	0.134	1.67	0.040	1.100	0.026	0.968	0.023
27	RRLJ (Mz-1)	Muzaffar Nagar**	1.24	8.47	0.203	4.31	0.103	1.82	0.043	0.98	0.023

* Sample obtained from Jammu & Kashmir.
† Sample obtained from Himachal Pradesh.
‡ Sample obtained from Delhi.
§ Sample obtained from Haryana.
** Sample obtained from Uttar Pradesh.

finally, it was an isocratic elution at 90:10 for 5 min. The total analysis time was 60 min. Mobile phase was delivered at a flow rate of 1 mL/min. The marker compounds were quantitated by using the external standard method.

Liquid chromatography–UV–DAD analysis

Using the described conditions, reproducible separations were achieved. Figure 5 shows the liquid chromatography (LC)–UV–DAD chromatogram (215 nm) of four marker compounds (Figures 1–4). All the marker compounds could be well separated, and the marked differences in their retention times made their quantitation easier. Figure 6 shows the LC–UV–DAD chromatogram of the sample where the presence of all the four markers has been observed.

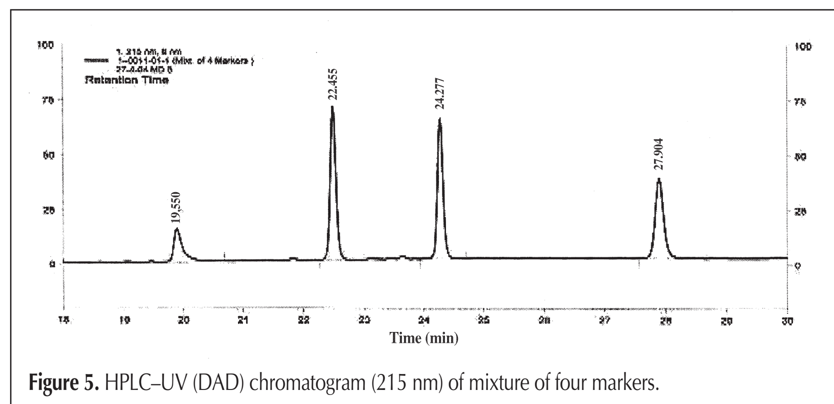

Figure 5. HPLC–UV (DAD) chromatogram (215 nm) of mixture of four markers.

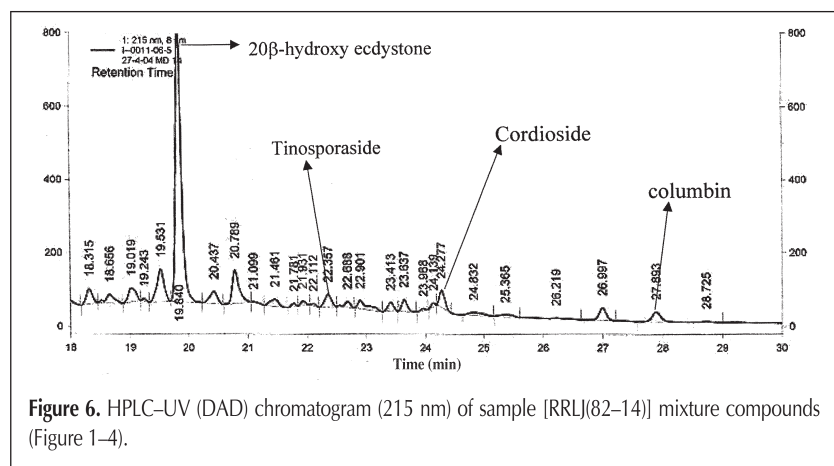

Figure 6. HPLC–UV (DAD) chromatogram (215 nm) of sample [RRLJ(82–14)] mixture compounds (Figure 1–4).

Results and Discussion

A simple reversed-phase HPLC–UV–DAD method, with gradient elution, was developed in order to qualitatively and quantitatively determine the four markers in plant extracts of *Tinospora sps*. A typical HPLC–UV–DAD chromatogram (Figure 6) was obtained using gradient elution of a crude extract, which exhibited a clean and smooth baseline with excellent resolution where all the marker peaks could be identified. The compounds such as 20β-hydroxyecdysone, tinosporaside, cordioside, and columbin exhibited retention times of 19.872, 22.443, 24.213, and 27.627 min, respectively. The peak purity was investigated by carefully studying the photo diode array details of all the peaks of interest. No indications of the impurities could be found. By measuring peak areas, calibration curves were constructed, which were linear (r^2 > 0.999) in the concentration range of 100 to 2000 ng. Two control standards containing 20 and 180 ng/µL of each of the markers in two different solutions were used (10 µL injection each) to ensure the accuracy and precision, where RSD (%) values were within 2% of the actual concentrations. This confirmed the precision and validation of the method. The method has the advantage of using a simple gradient elution in the reverse phase without adding buffers.

The levels of four marker compounds in the three species of Tinospora have been determined (Table I–III). The studies have revealed that *T. cordifolia* species possess efficient levels of all the four bioactive compounds (Table I) followed by *T. crispa* (Table II) and then the *T. malabrica* species (Table III). Furthermore, it has been found that

Table II. Amounts of Marker Compounds (Figure 1–4) in *T. crispa spp.*

S. no.	Accession code	Origin	Weights of extracts in grams	20β-hydroxy -ecdysone		Tinosporaside		Cordioside		Columbin	
				Extract (%)	Plant (%)	Extract (%)	Plant (%)	Extract (%)	Plant (%)	Extract (%)	Plant (%)
1	RRLJ (C1)	Kaziranga Natl. Park*	1.503	3.91	0.117	0.201	0.006	0.563	0.016	0.711	0.021
2	RRLJ (C2)	Dibrugrah*	1.302	0.816	0.021	0.537	0.013	0.389	0.010	0.088	0.002
3	RRLJ (C3)	Johrat*	1.414	0.463	0.021	0.101	0.002	0.077	0.002	0.101	0.002
4	RRLJ (C4)	Dispur*	1.406	0.617	0.12	0.806	0.022	0.432	0.012	0.32	0.009
5	RRLJ (C5)	Tuli*	1.381	0.943	0.02	0.544	0.014	0.469	0.012	0.109	0.002
6	RRLJ (C6)	Karimgang*	1.361	0.802	0.02	0.30	0.007	0.08	0.002	0.125	0.003
7	RRLJ (C7)	Kokrajar*	1.370	0.643	0.016	0.204	0.005	0.732	0.019	0.62	0.016

* Sample obtained from Assam.

Table III. Amounts of Marker Compounds (Figure 1–4) in *T. Malabarica spp.*

S. no.	Accession code	Origin	Weights of extracts in grams	20β-hydroxy -ecdysone		Tinosporaside		Cordioside		Columbin	
				Extract (%)	Plant (%)	Extract (%)	Plant (%)	Extract (%)	Plant (%)	Extract (%)	Plant (%)
1	RRLJ (M1)	Cochin*	1.000	0.196	0.003	0.097	0.001	0.117	0.002	–	–
2	RRLJ (M2)	Thriuvanthapu-ram*	1.290	0.208	0.005	0.302	0.007	0.112	0.002	0.824	0.001
3	RRLJ (M3)	Calicut*	1.100	1.486	0.035	0.147	0.003	0.162	0.003	0.154	0.003
4	RRLJ (M4)	Ernakulam*	1.300	1.069	0.027	0.110	0.002	0.115	0.003	0.84	0.002
5	RRLJ (M5)	Palakkad*	1.220	0.424	0.101	0.242	0.005	0.079	0.001	0.107	0.002
6	RRLJ (M6)	Kannur*	1.184	0.543	0.11	0.320	0.007	0.102	0.002	–	–

* Sample obtained from Kerala.

accession RRLJ (82-14) (Table I) collected from Ram Nagar (Udhampur Dstt.), Jammu, and Kashmir state (India) can be an excellent source for 20β-hydroxyecdysone and columbin. However, accessions RRLJ (82-12) (Table I) collected from Bakshi Nagar (Jammu District, Jammu and Kashmir, India) was found to possess efficient levels of cordioside and columbin. Both these plants occur widely in areas adjoining to the Jammu province of Jammu and Kashmir (North India) state, which is at an altitude of 1,112 meters above mean sea level (AMSL)

It was also found that RRLJ (M1) and RRLJ (M6), accessions of *T. malabarica* (Table III) collected from the southern region of India, lacked the marker compound columbin. Further, it was found that all six accessions of the same species contain poor concentrations of all the marker compounds. The reason for the low concentration of the marker compounds may be because of the existence of these species at lower altitudes (i.e., only 50 meters AMSL). Our results showed that the *T. crispa* species (Table II), which is found in the eastern parts of India, located at a hight of 580 meters AMSL, possesses a smaller concentration of all the marker compounds when compared with *T. cordifolia*.

Our broad-based studies revealed a high level of variation of the four secondary metabolites among the three species, which may be because of the wide distribution of Tinospora species in different eco-geographical zones of the country. The results obtained by chemoprofiling showed a high level of correlation with our molecular data of the species. Both the accessions [i.e., RRLJ (82-12) and RRLJ (82-14)] were well separated by randomly amplified polymorphic DNA analysis. Further, the accessions RRLJ (M1) and RRLJ (M2) did not cluster with the rest of the species of *T. malabarica* by restriction fragment length polymorphisom of internal transcribed spacers of ribosomal DNA analysis (20)

Conclusion

A requirement of our research program was to evaluate a large number of samples of Tinospora species by HPLC and to correlate these results with the genomics of different species/accessions to identify the species within a particular region, expressing the appreciable amounts of all the four pharmaceutically important biomarkers. It was important to develop an easy, reproducible, and rapid HPLC method for the separation and quantitative (%) evaluation of the four markers of all the three species of Tinospora sps. In conclusion, a novel, sensitive, and specific method has been developed to quantitate the four marker compounds simultaneously. The assay is quite reliable and selective and has been fully validated. The method has been applied to quantitate four compounds in three different species of Tinospora. The results indicate that the developed method can be readily utilized for quality control of the Tinospora herb.

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