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Original Article

Phytochemical composition changes in untreated stem juice of *Tinospora cordifolia* (W) Mier during refrigerated storage

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ARTICLE INFO

Article history:
Received 17 November 2012
Accepted 18 January 2013
Available online 13 February 2013

Keywords:
Tinospora cordifolia
Alkaloids
UPLC–QTOFMS
Phytochemical profile
Degradation

ABSTRACT

Background: Juice of *Tinospora cordifolia* is well reported for its immuno-modulatory and adaptogenic properties. Importance of the plant is well understood as its stem juice was widely used during the Swine flu break in India as an immuno-modulator. On the contrary, there are no data available on the shelf-life of juice. Therefore, effects of cold storage on the phytochemical profiles of *T. cordifolia* stem juice were analyzed.

Methods: Juices of mature stems of the *T. cordifolia* were prepared and stored at 0 °C. The juice samples were analysed on 0, 15 and 30 days intervals using UPLC–QTOFMS. The juice samples were resolved on C-18 (4.6 × 250 mm, 1.8 μm) column at 40 °C. The optimized method provided a good linear relation ($r \geq 0.9213$ for all the internal standards), satisfactory precision (RSD values less than 2.1%) and good recovery (97.3–101.2%).

Results and discussion: Initial analysis of MS^n data showed presence of 14,101 molecular features in the samples. Variation in the molecular features analysed using principal component analysis and discrimination models showed 99% variation across the samples analysed on 0, 15 and 30 day. Jatrorrhizine, mangoflorine, menisperine, columbamine, berberine and tinosporoside along with other most abundant metabolites were degraded 70–80% over a period of 15 days and 99% in 30 days. High degradation rate of metabolites constituents was observed may be due to the enzymes present in the juice or oxidative reactions. Therefore, it is recommended to use the fresh juice of *T. cordifolia* and stability of its herbal formulation should be analysed carefully.

1. Introduction

Change in the concentration of phytochemicals due to degradation during storage can greatly compromise the quality of herbal drugs. As some of the herbal drugs prescribed in form of juice (called Sawras in Ayurveda) therefore, effect of storage temperatures need to be studied. Thermal processing, which is required to inactivate microorganism and enzymes present

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0974-6943/$ – see front matter
http://dx.doi.org/10.1016/j.jopr.2013.01.018

in the juice, negatively affects the sensory and nutritional compounds of plant-based foods. Therefore, to retain original medicinal and nutritious values non-thermal technologies and their impacts are needed to be analysed. In the few past years, the emerging field of metabolomics has become an important strategy in many research areas such as diseases diagnostics,[1] drug discovery,[2] human nutrition,[3] and also, as recently reviewed in the food science, where it was used for informative, discriminative, and for predictive purposes associated with food quality and safety.[4] Now software capable of rapid data mining and aligning algorithms for processing of large spectral data sets of metabolome is available.[5–7] Advanced chemometric tools for reduction of data dimensionality present in obtained multivariate records are useful tools. Principal component analysis (PCA) represents initial exploration of data internal structure and sample clustering. Along with PCA linear discriminant analysis, partial least squares discriminant analysis (PLS-DA), support vector machine or artificial neural networks enables classification of samples from the molecular features present in them.[8]

In the current study, high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC–QTOFMS) has been used for non-targeted analysis of phytochemical profile modification during refrigerated storage of untreated stem juice of *T. cordifolia*. *T. cordifolia* (W) Mier (Menispermaceae), is referred to as "nectar of immortality" and "heavenly elixir" and a well known plant for its traditional medicinal properties. The importance of the plant can be understood by its very wide use and coverage in Indian news papers during the Swine breakthrough in the India. This shrub is well reported for its immuno-modulator and adaptogenic properties.[9–11] It is a popular ingredient in many formulations in various forms such as juice, paste, prepared starches, powders and decoctions which are used as antioxidant,[12] anti-cancer,[13] anti-inflammatory,[14] anti-diabetic[15] and special decoctions in gouts and rejuvenating tonic.[16] It is the main drug of choice for hepatic aliments.[17] Syringin and cordiol inhibited the *in vitro* immunohaemolysis due to inhibition of the C3-convertase of the classical complement pathway. Humoral and cell-mediated immunity were also dose-dependently enhanced. Macrophage activation was reported for cordioside, cordiofolioside A and cordiol.

A very few studies have reported the impact of refrigeration and time on the juices of medicinal plants on the degradation of bioactive compounds. In present study, UPLC–QTOFMS data of *T. cordifolia* juice was analysed by commercially available software packages to obtain PCA and PLS-DA at different time intervals.

2. Material and methods

2.1. Plant material

Stems of same diameter of four year old *T. cordifolia* Miers (protected from the use of any type of pesticides) were collected from Medicinal Plant Garden of NRIBAS, Pune. The samples collected during rainy season were authenticated by Dr GB Rao and preserved as Voucher No. 296 in herbarium.

2.2. Chemicals

Standard compounds lidocaine, D-camphor, 5, 7-isoflavone and berberine were purchased from MP Biomedicals (each of purity ≥99%). Acetonitrile, formic acid and water of LCMS grade were purchased from Sigma–Aldrich.

2.3. Sample preparation

Stems of *T. cordifolia* were washed with deionized water. The juice of 15 g stem sample was extracted with 15 ml deionized water (Direct-Q, Millipore) at 25 °C and centrifuged at 15,000 g for 10 min at 4 °C temperature to remove debris. Equal volumes of juice and ethanol were mixed and kept in −80 °C for 5 h to ensure complete protein precipitation and centrifuged at 15,000g for 10 min at 4 °C temperature to remove protein precipitates. Lidocaine (234.3*m/z*) and 5, 7-isoflavone (284.3 *m/z*) were infused with samples as standard markers. The juice samples were stored at 4 °C till further use.

2.4. UPLC–QTOFMS

The chromatographic separation of *T. cordifolia* stem juice was carried out using Zorbax Eclipse Plus reversed phase C18 column (250 mm × 2.1 mm internal diameter) of 1.8 μm particle sizes on Agilent 1200 Series UPLC interfaced to an Agilent 6520 Accurate-Mass QTOFMS. A volume of 20 μl of each sample was injected by auto-sampler to the column. Mobile phase comprised solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was used in gradient mode. The following gradient elution was carried out: eluent B 5–20%from 8 to 15 min; eluent B 45–65% from 22 to 30 min; eluent B 65–90% from 35 to 40 min (to wash the column); eluent B 5% for 40–45 min (for column equilibration). The flow rate of the solvent was maintained 0.2 ml/min. The mass spectrometer was operated in positive mode in the *m/z* range 100–1100 at acquisition rate of 2 MS/MS and 3 MS spectra/s with following parameters: gas temperature 350 °C, nebulizer 45 psi, drying gas flow 11 L/min, capillary 3.5 V, skimmer voltage 65 V and fragmentor voltage 175 V. Instrument was calibrated and tuned as per instruction of manufacturer. To assure mass accuracy of recorded ions, continuous calibrations with internal and infused standards with samples (lidocaine, D-camphor, 5, 7-isoflavone) were performed during analysis.

2.5. Data processing

MassHunter Workstation software (MassHunter version 3.1) was used for UPLC–QTOFMS data processing which includes of peak detection, chromatographic alignment, background removal, normalization and mass filtering. The raw data set acquired were initially analyzed by Molecular Features (MFs) extraction software for the detection of the compounds. The list of chemically qualified MFs was generated by eliminating interferences and reducing data complexity. Molecular formulae were estimated on the basis of fragment patterns of ions. Different intensity threshold from 1000 to 10,000 cpu was used for molecular feature extraction in the full retention time range.

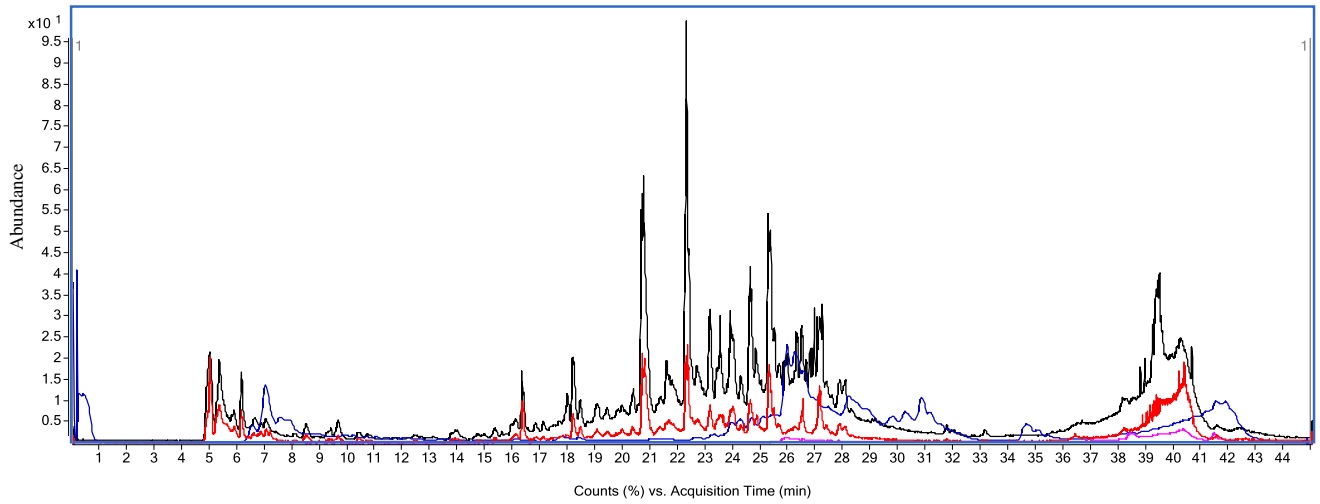

Fig. 1 – Total ion chromatograms of the samples analysed. Blank, Group 1, 2 and 3 are represented by Pink, Black, Red and Blue colours respectively.

2.6. *Statistical analysis*

Background subtracted data of compound exchange (.cef) files was exported into the Mass Profiler Professional (MPP) software package (Agilent Technologies, version B 02.02). MPP was used for statistical evaluation of technical reproducibility and comparison of samples. In MPP, the retention time and *m/z* alignment across the sample sets was performed using a tolerance window of 0.2 min and 20 mDa. Molecular Features were reduced stepwise based on frequency of occurrence, abundance of respective MFs in classes and one-way analysis of variance (ANOVA). A probability level of $p < 0.05$ was applied to reduce nonsignificant molecular features. Compounds that satisfied fold change cut-off 2.0 in at least one condition pair were selected for further analysis and differentiation. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using MPP.

3. Results and discussion

3.1. *Method development*

The MS/MS were performed in positive ion mode with optimized parameters. As juice of *T. cordifolia* has been used directly in medical situations, therefore, to avoid any discrimination of metabolite present in the samples any kind of pretreatment was avoided. The total ion chromatogram of the juices showed visible changes in the profiles at different time intervals and least peaks in the sample studied after interval of one month (Fig. 1). Chromatographic peaks with base width of 15 s were obtained gave approximate separation peak capacity of 4 peaks per minute. Retention time (RT) variability across the samples was calculated using the infused standards and found to be 2 s and a relative standard deviation of less than 5%.

For metabolomics studies TOFMS is an effective tool due to accurate mass accuracy less than 5 ppm and higher resolution. The instrument employed in the current study was utilizing 2/4 GHz analogue to digital converter offering high dynamic range and minimizing threat of saturation. Furthermore, TICs in Fig. 1 showing metabolite fingerprints clearly indicates the shift in the peaks of spectra recorded after 15 days and 30 days intervals, shows that the degradation rate is very high in the samples stored at 0 °C.

3.2. *Qualitative analysis*

Automated extraction of ions using algorithm showed presence of 14,101 molecular features in the samples. Isotopes and

Table 1 – The overview of molecular features (MF) extracted at various intensity threshold settings and applied filtering steps in positive ionization mode.

Intensity threshold setting (cps)	Number of MFs across the sample set			
	Initial[a]	Filtering by frequency[b]	One-way ANOVA[c]	Filtering by fold changed (fold change ≥2)
1000	16,820	865	225	25
5000	14,101	808	126	26
10,000	8213	640	46	14

a After removal of MFs extracted from blank samples.
b Filtering criterion was the presence of a MF in at least 50% of samples at least in one group.
c The criterion to pass the filter was *p*-value <0.05.

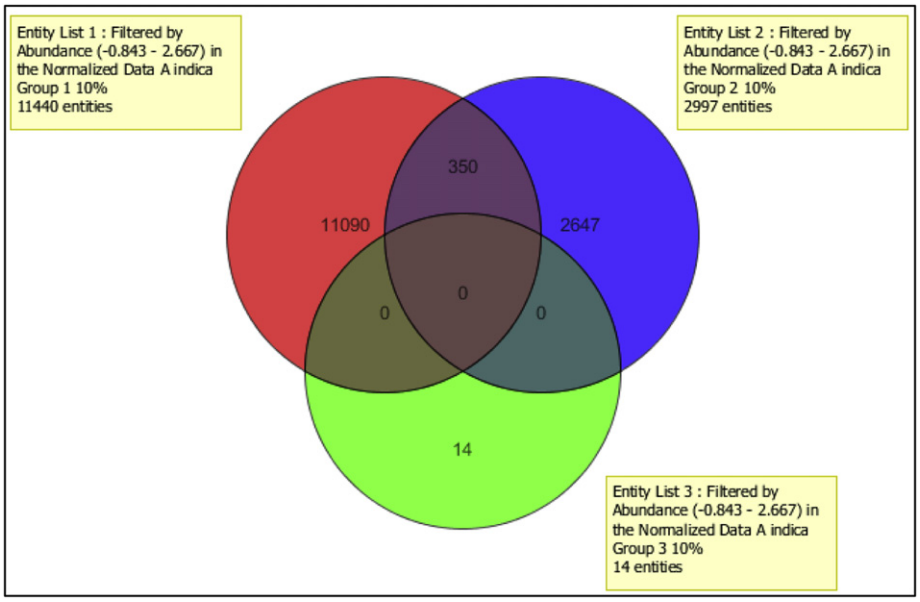

Fig. 2 – MS/MS extraction provides a larger number of unique molecular features in positive TOF-MS mode. The Venn diagram depicts the number of molecular features obtained under positive TOF-MS conditions at different time intervals using MPP software.

adducts were supposed to have identical elution profile and merged into molecular features as a single variable. Number of aligned molecular features can be influenced by intensity of threshold, therefore, a constant intensity threshold 5000 cps was employed to extract the data across the samples (Table 1). Various filters were applied in ensure quality of data shown in Table 1.

Venn diagram in Fig. 2 shows similar and differential molecular features in all the three groups. The degradation rate noticed was amazingly high and it is clear from the graphic representation that all the metabolites get degraded within one month. Merely 14 molecular features were observed in group at a threshold of 5000 cps. The results indicate the presence of enzymes in the juice which are active even at 0 °C. The confirmation this has been done by protein estimation of fresh juice which showed around 42% total proteins in the juice. For further confirmation of Venn diagram results, PCA and PLS-DA were taken into consideration.

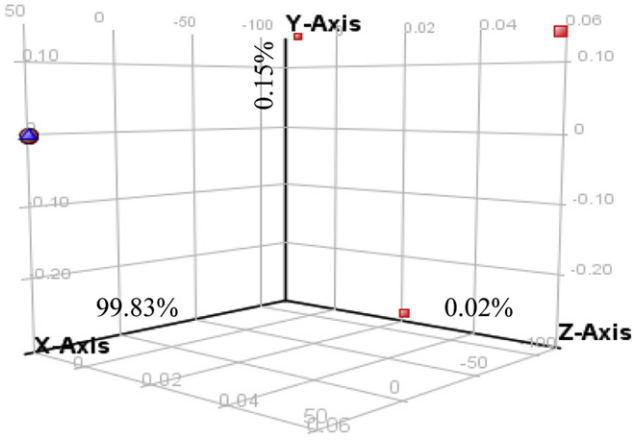

Fig. 3 – Principal component analysis of the TOF-MS spectra. PC1 vs. PC2 score plot of an unsupervised PCA of the positive ion mode mass spectra acquired at different time. Based on their distribution with respect to the *x*-axis, the samples can, qualitatively, be grouped into two clusters: (I) Zero time (Red); (II) and after 15 (Blue) and 30 (Brown) days time.

Table 2 – The overview of most abundant MFs obtained in positive ion mode at intensity threshold 50,000 cps.

S. no	RT	*m/z*	Formula	Mass (MFG)	Score (MFG)	Abundance
1.	7.028	448.19674	$C_{23}H_{29}NO_8$	447.18932	99.52	65,443
2.	7.045	120.08017	C_8H_9N	119.0735	97.54	77,389
3.	8.354	300.23688	$C_{10}H_{25}N_{11}$	299.22944	72.23	31,193
4.	8.997	205.09802	$C_{11}H_{12}N_2O_2$	204.08988	96.51	76,320
5.	9.012	188.13096	$C_9H_{17}NO_3$	187.12084	92.2	44,292
6.	9.106	522.21784	$C_{22}H_{35}NO_{13}$	521.21084	99.71	54,334
7.	19.78	561.19418	$C_{24}H_{28}N_6O_{10}$	560.18669	99.04	44,332
8.	22.201	311.12898	$C_{20}H_{14}N_4$	310.12185	96.82	46,224
9.	22.238	343.15536	$C_{21}H_{18}N_4O$	342.14806	96.41	68,654
10.	22.264	685.30007	$C_{37}H_{36}N_{10}O_4$	684.2921	99.33	64,211
11.	22.328	540.24361	$C_{26}H_{37}NO_{11}$	539.23666	96.38	71,217
12.	22.519	545.19965	$C_{24}H_{28}N_6O_9$	544.19178	98.53	58,975
13.	24.872	491.31369	$C_{28}H_{38}N_6O_2$	490.30562	98.88	32,213
14.	24.913	469.33146	$C_{30}H_{44}O_4$	468.32396	94.53	57,282
15.	24.925	235.17038	$C_{15}H_{22}O_2$	234.16198	95.25	89,895
16.	24.973	217.1598	$C_{15}H_{20}O$	216.15142	93.25	104,380
17.	25.348	743.30335	$C_{38}H_{42}N_6O_{10}$	742.29624	99.06	61,605

Table 3 – Mass fragmentation of identified compounds.

S. no	Retention time (min)	*m/z*	MS/MS, *m/z*	Identification
1	21.80	338.14	323.114, 308.09, 294.112	Jatrorrhizine
2	14.48	342.17	297.113,282.08, 265.08, 233.05, 205.063	Mangoflorine
3	16.90	356.19	311.127, 279.10, 265.08, 236.08	Manisperine
4	22.18	338.13	323.1, 308.09, 294.1, 279.08	Columbamine
5	28.06	337.07	320,306, 292	Berberine
6	19.90	431.17	234.02	Tinosporide

3.3. *Chemometric analysis*

PCA transformations are helpful to visualize the most significant differences in the mass profiles between samples and allow similar samples to be grouped together. The first principal component along X axis is most strongly influenced by the combination of ion signals that exhibit the largest change between the recorded spectra. In the present case, it was found to be 99.83%. Fig. 3 shows the score plot of the unsupervised PCA. Group 1 (fresh juice sample) was found to be very different and contains highest number of molecular features. Molecular feature represented in PCA plot in group 1, 2 (juice sample after 15 days storage) and 3 (juice sample after 15 days storage) were observed to be 11,271, 2996 and 14 respectively, suggests the high degradation rate in metabolites of *T. cordifolia* even after storage at 0 °C.

The most abundant metabolites which can also be used a markers, extracted with a threshold 50,000 cps from the fresh juice are listed in Table 2. It wasn't feasible to select a marker compound for 3rd group for subsequent tentative identification. Therefore the compounds present in Group 1 and 2 were used to compare degradation rate based on the marker compounds. For formulae generation, the isotopic pattern of unknown compound, relative high atom number and low mass error limits were used. Based on these factors MassHunter software generated several formulae which has

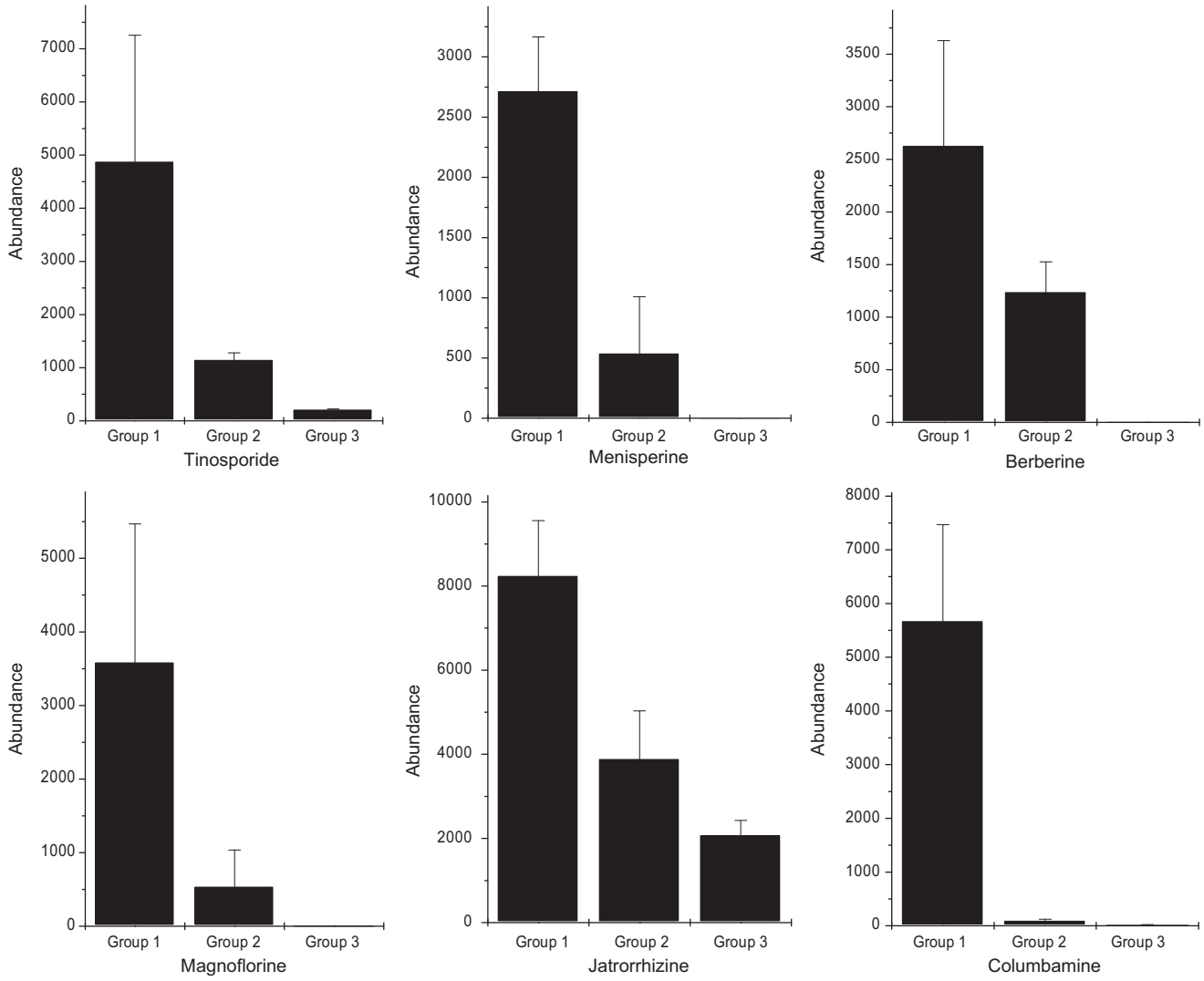

Fig. 4 – Abundances of identified compounds in different groups showing different degradation rates.

Table 4 – The overview of classification result obtained by PLS-DA model.

	Group 1	Group 2	Group 3	Accuracy (%)
Group 1	3	0	0	100
Group 2	0	3	0	100
Group 3	0	1	2	66.666
Discrimination ability (%)				88.888

been sorted out by MGF score. Molecular formulae presented in Table 2 (along with predicted abundances) and 3 had the highest score and lowest error calculated by the software. A compound search for the above candidates was performed using online databases and available literature. The metabolites which were identified by comparing standard mass spectra and fragmentation pattern and found only in fresh juice are given in Table 3. Degradation rate of important and known metabolites were explored using total abundance of metabolites present in different sample (Fig. 4).

A supervised pattern recognition method was used to discriminate and classify the stem juice samples. The result in terms of classification abilities of the samples showed 88.888% accuracy (Table 4). The classification ability was observed to be slightly lower due to incorrect assignment of one sample of Group 3 in may be due to extensive degradation in Group 2. The same has been confirmed by comparing the abundances of ions of identified compounds in juice (Fig. 4) where Group 2 showed very low abundance as compared to Group 1.

4. Conclusions

The UPLC–QTOFMS is advanced technique used extensively for diseases diagnostics, drug discovery and human nutrition. In this study, the technique has been successfully used to explore the stability of untreated stem juice of stems of *T. cordifolia* stored at 0 °C. The reported medicinally important compounds i.e. jatrorrhizine, mangoflorine, manisperine, columbamine, berberine and tinosporoside were identified using standard mass spectra from literature and comparing the mass fragmentation patterns. Manisperine is the alkaloid, first time reported from *T. cordifolia*. There abundance comparisons showed complete degradation of some compounds after one month storage. As consumers continue to seek products with improved medicinal value and functionality, the stabilizers for medicinal juices should be used judicially. It is also advisable to use the fresh juice of *T. cordifolia* instead of stored one, as degradation starts immediately in the juice contents even if stored at 0 °C. At the same time, considering the encouraging results obtained in this study, the application of UPLC–QTOFMS to detect stability of herbal products seems to be a very promising approach.

Conflicts of interest

All authors have none to declare.

Acknowledgements

Authors are thankful to CCRAS, Department of AYUSH, Government of India to support the study.

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