Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

A Metabolomics Approach to Profile Novel Chemical Markers for Identification and Authentification of *Terminalia* Species

Bharathi Avula,[1] Kate Yu,[2] Yan-Hong Wang,[1] Jordan Blodgett,[2] Alan Millar,[2] and Ikhlas A. Khan[1,3,4]

1. National Center for Natural Products Research, School of Pharmacy, University of Mississippi, MS, U.S.
2. Waters Corporation, Milford, MA, U.S.
3. Department of Pharmacognosy, School of Pharmacy, University of Mississippi, MS, U.S.
4. Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

APPLICATION BENEFITS

First metabolomics approach for a comprehensive chemical profiling of the *Terminalia* species using UPLC®/TOF MS^E, coupled with Multivariate statistical analysis (MSA), and applying the same approach to authenticate a popular Ayruveda medicine, Triphala.

WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® G2 QTof Mass Spectrometer

MarkerLynx XS™ Application Manager

MassFragment™ Application Manager

KEY WORDS

UPLC, QTof MS^E, Multivariate Statistical Analysis, plant metabolomics, plant species authentication, *Terminalia*, Traditional Medicine, Ayruveda medicine, triphala

INTRODUCTION

Terminalia is a genus of large trees of the flowering plant family Combretaceae, which is comprised of around 100 species that are distributed in tropical regions around the world.[1] Figure 1 shows the different *Terminalia* species that were included in this experiment. The fruits and bark from various *Terminalia* species have been used in Traditional Indian Medicine since ancient times for the treatment of various ailments. Some species have been used in Ayurvedic formulations, in either a single herb formulation, or in multiple herbal formulations.[2]

Terminalia species provide rich sources of secondary metabolites, including cyclic triterpenes and their derivatives, flavonoids, tannins, and phenolic acids.[3] The exact chemical classes and levels may vary in different *Terminalia* species, which may contribute to the different biological activities observed. Yet mixtures of multiple species of *Terminalia* show other health benefits versus their individual effects. The Ayruveda medicine triphala is a perfect example of such a case.

To date, Thin Layer Chromatography (TLC) is the common QC method used for analysis of triphala, and gallic acid and ellagic acid are used as the marker standards. However, gallic acid and ellagic acids are present in a majority of the *Terminalia* species, including the three fruits used in triphala, making them less than ideal for use as markers for authentication of triphala. It is important to have a suitable analytical method to distinguish individual herbs that will allow adequate quality control (QC) of raw materials and standardization of finished products.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC HSS T3 2.1 x 100 mm, 1.8 µm
Column temp.:	60 °C
Flow rate:	500 µL/min.
Mobile phase:	A: water + 0.1% formic acid B: methanol
Injection volume:	5 mL
Gradient:	80% A to 40% A for 29 min., then step to 0% A and hold for 3 min. before re-equilibration
Total cycle time:	35 min.

MS conditions

MS system:	Xevo G2 QTof Mass Spectrometer
Ionization mode:	ESI– and ESI+
Acquisition range:	50 to 1200 *m/z*
Capillary voltage:	3 kV
Cone voltage:	30 V
Desolvation temp.:	500 °C
Desolvation gas:	900 L/Hr
Source temp.:	120 °C
CE:	Low: 4 eV High: 45 to 60 eV

The UPLC/oaTOF MS^E analysis was performed for multiple *Terminalia* samples, shown in Table 1 in both positive and negative ionization modes. In this study, the results obtained in negative ionization mode were the main focus. To ensure data integrity, all samples were pooled into a single vial and used in the QC run. For each individual sample, six replicates of injection were performed with the sequence of the injections randomized.

Sample preparation

In order to perform the determinations for commercial products, five capsules were weighed and opened, and the contents were emptied. The content of capsules were mixed and triturated using a mortar and pestle. Dry plant samples (0.5 g), or an adequate amount of capsule/powder content, were weighed (about 500 mg) and sonicated in 2.5 mL of ethanol for 30 min, followed by centrifugation for 15 min at 4000 rpm. The supernatant was transferred to a 10-mL volumetric flask. The procedure was repeated three times, and the respective supernatants were combined. The final volume was adjusted to 10 mL with ethanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.2 µm nylon membrane filter. The first 1.0 mL was discarded and the remaining volume was collected in an LC sample vial. Each sample solution was injected in triplicate.

RESULTS AND DISCUSSION

The UPLC/*oa*TOF MS^E/MSA was performed for a total of 15 samples, as shown in Table 1. The key separation factors were the resolution and the peak capacity. Methanol was chosen over acetonitrile as it appeared to better retain peaks. Figure 2 shows the Base Peak Ion (BPI) chromatogram comparison for the five different herbal extracts, plus the two commercial products. The differences of these samples are shown in Figure 1.

NCNPR Accession #	Name	Place
7523	*Terminalia chebula*	CRISM, INDIA
7786	*Terminalia chebula*	CHINA
7787	*Terminalia chebula*	CHINA
8207	*Terminalia arjuna*	CRISM, INDIA
4995	*Terminalia arjuna*	INDIA
1229	*Terminalia arjuna*	INDIA
7504	*Terminalia bellerica*	CRISM, INDIA
4992	*Terminalia bellerica*	CRISM, INDIA
4993	*Terminalia bellerica*	CRISM, INDIA
2518	*Emblica officinalis*	HAMDARD UNIVERSITY, PAKISTAN
7795	*Emblica officinalis*	COMMERCIAL
4923	*Emblica officinalis*	CRISM, INDIA
1356	*Terminalia species*	COMMERCIAL
	Product-1	Dosage Form: Capsules
	Product-2	Dosage Form: Powders

Table 1. List of the 15 samples analyzed for this experiment.

Figure 1. E. officinalis and Terminalia species that were included in this work.

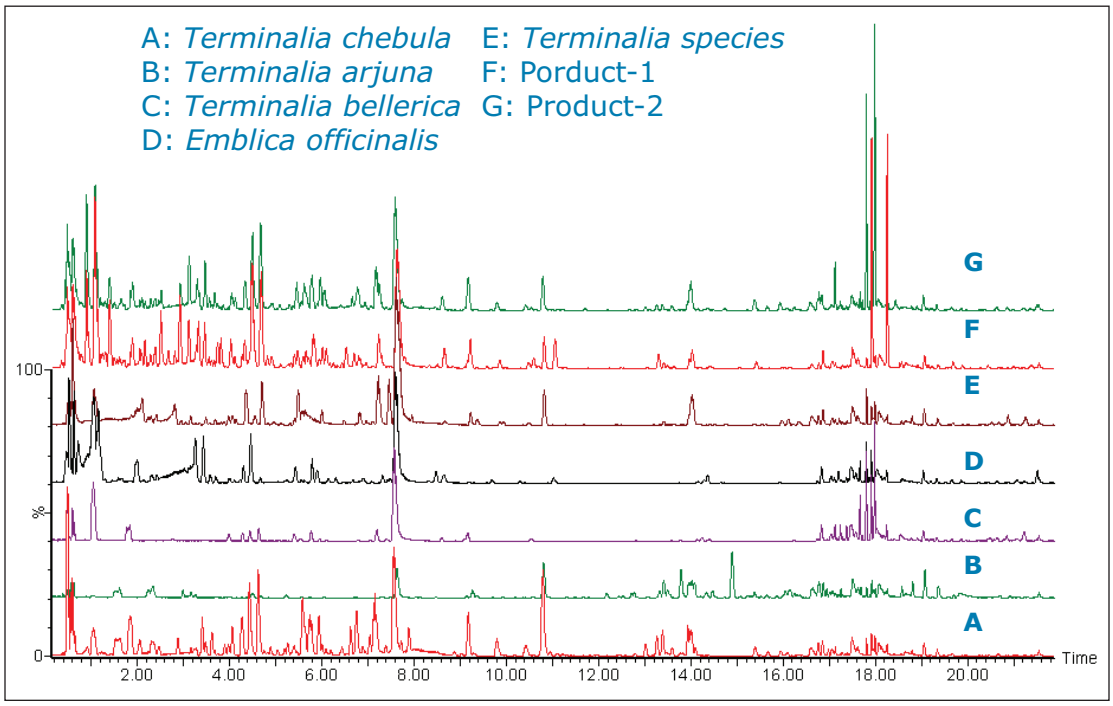

Figure 2. BPI comparison of the different species, plus two commercial Terminalia products.

A multivariate statistical analysis tool was used to obtain the comprehensive chemical profile of the samples. For multi-sample group analysis, Principle Component Analysis (PCA) is an effective tool. When performing MSA analysis, each sample group needs to be injected no less than three times to ensure the statistical validity (N=6 for this analysis); and a QC sample must be prepared for the system suitability test. In this study, all 15 samples were pooled into a single sample and used as the QC sample.

Figure 3 shows the PCA scores plot for the entire dataset. There is a clear grouping pattern among different species of the herb. All "old" samples (collected and stored for more than two years), showed clear differences from the fresh samples. These differences seem to be more significant than the differences caused by various plant locations. The *Terminalia* species showed a closer similarity to *Terminalia chebula* than to *Terminalia bellerica* and *Terminalia arjuna*. *Embelica officinalis* was significantly different from all of the *Terminalia* spp, which was somewhat expected. The tightness of all of the QC injections provided a good sign of the system stability.

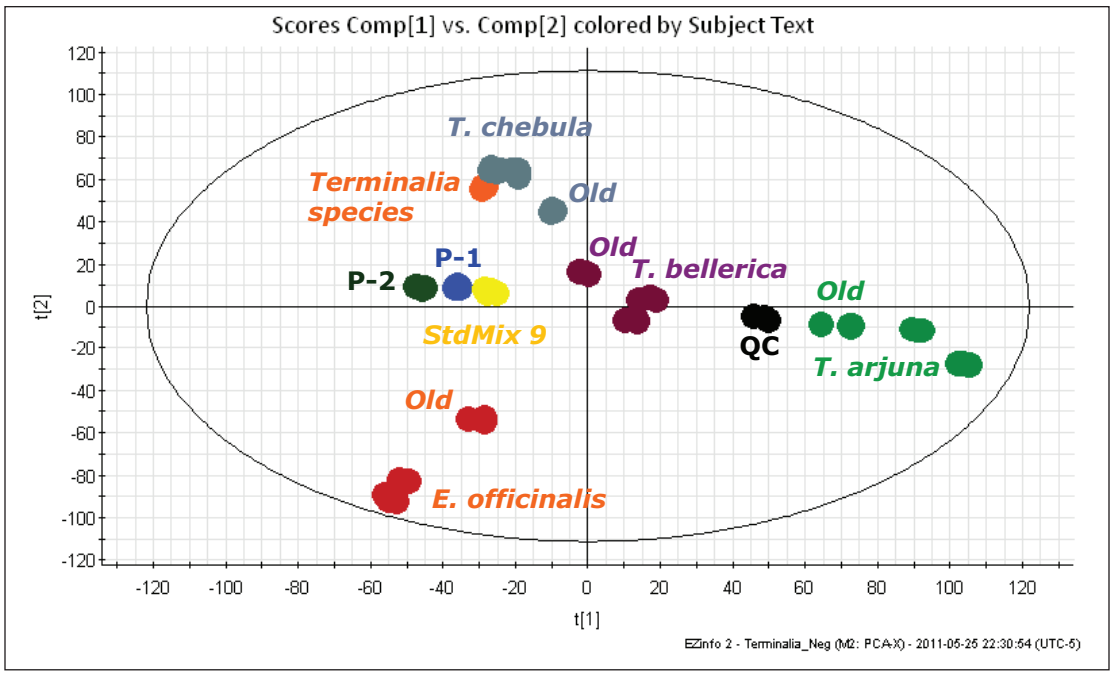

Figure 3. The PCA scores plot for the entire sample set including the QC.

Figure 4 shows the loadings plot that correlates to the scores plot from the same PCA analysis, which highlights the key markers – exact mass retention time (RT) pairs – that have the most significant contributions for the sample grouping. The geographic distribution of the markers from the loadings plot correlated to the geographic distribution of the sample groups on the scores plot shown in Figure 3. For example, the two markers highlighted in Figure 4 are closely related to the sample groups *T. arjuna* and the StdMix-9. Hence, the markers in this region were expected to be higher in content in these two groups of samples. And the markers in the similar location were expected to have similar trending plots.

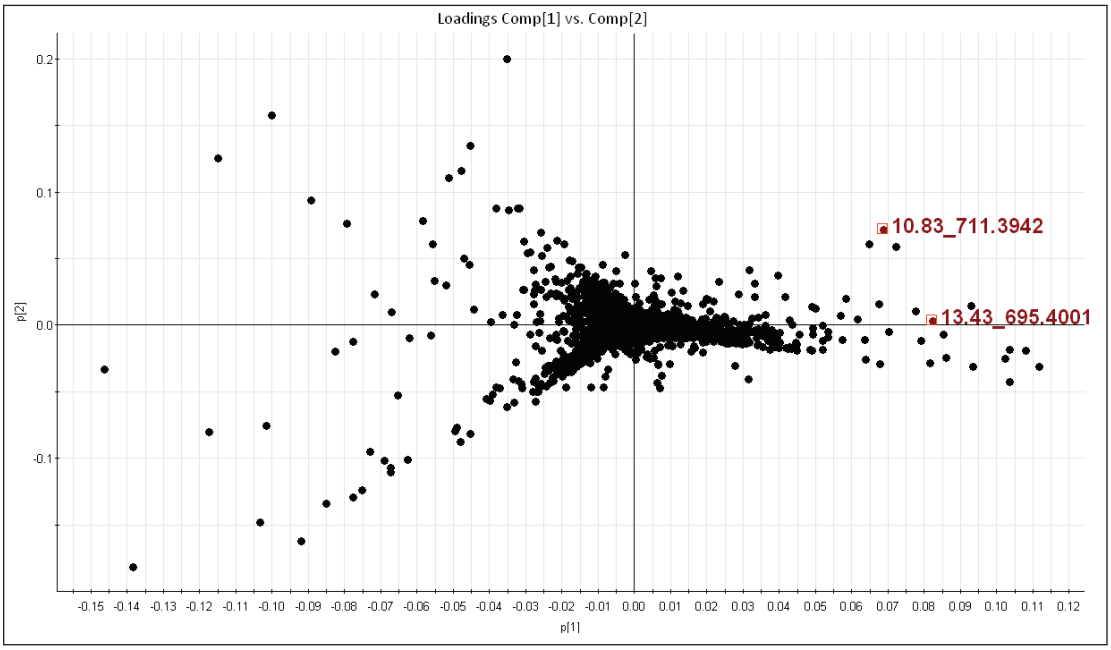

Figure 4. PCA loadings plot of the entire Terminalia data set.

Figure 5 shows the trending plot for the two markers shown in Figure 4. The trends of these two markers are identical from sample to sample. As expected, they were high in *T. arjuna* and in StdMix9. The StdMix-9 is a mixture of nine markers that were known from previous studies. As it is standard in pure solvents, it is not surprising to see these two markers were very high in content. These two markers were also high in *T. chebula*. However, there are other markers more dominating in *T. chebula*. This finding provides an explanation as to why the markers were relatively distant from the *T. arjuna* group from the PCA scores plot.

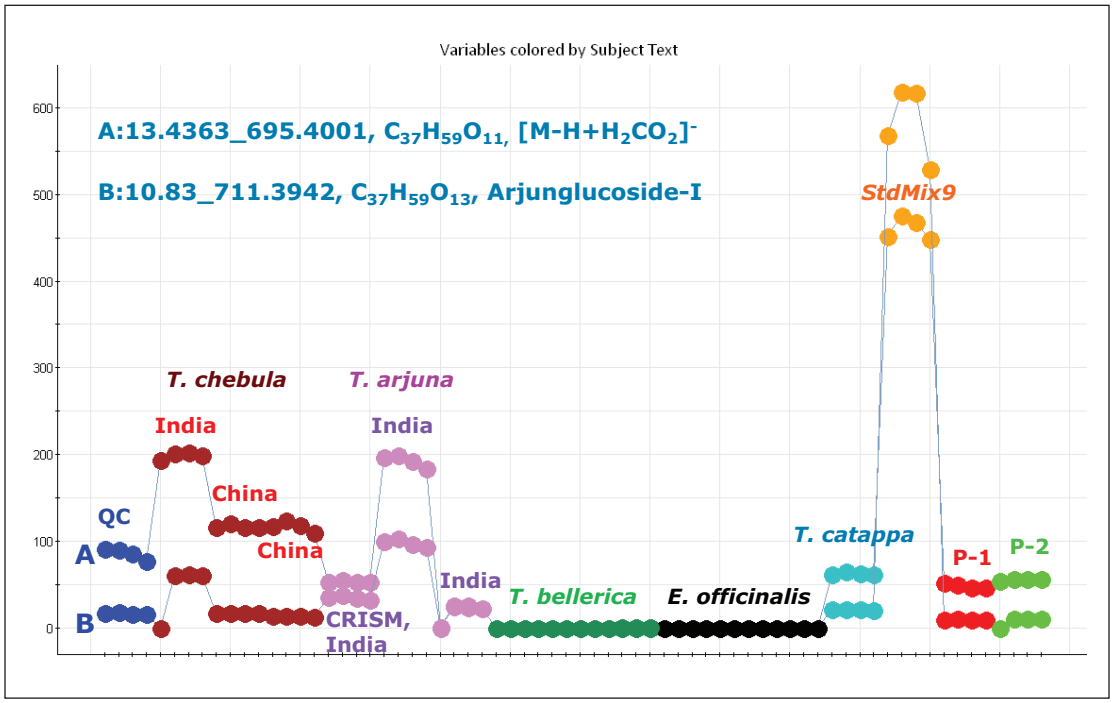

Figure 5. Trend plot of the two key markers labeled from the PCA loadings plot shown in Figure 4.

Also observed from the Trend plot was the fact that these two markers were significantly higher in content in the samples that were harvested in India. Using *T. chebula* as an example, the contents of these two markers were significantly higher in the India sample than the two samples from China, which suggests that plant location has a more profound influence in chemical content.

Terminalia is also a popular herb in Traditional Indian Medicine. For example, the Ayurveda medicine, triphala is a remedy that calls for the mixing of *T. chebula*, *T. bellerica*, and *Emblica officinalis* in equal portions. The authentication of triphala can become a challenge. The two commercial products (P-1 and P-2) in our sample set for this project are indeed the triphala extract. Figure 6 shows the PCA scores plot of the two products, plus the other herbs that the formula calls for: *T. chebula*, *T. bellerica*, and *Emblica officinalis*.

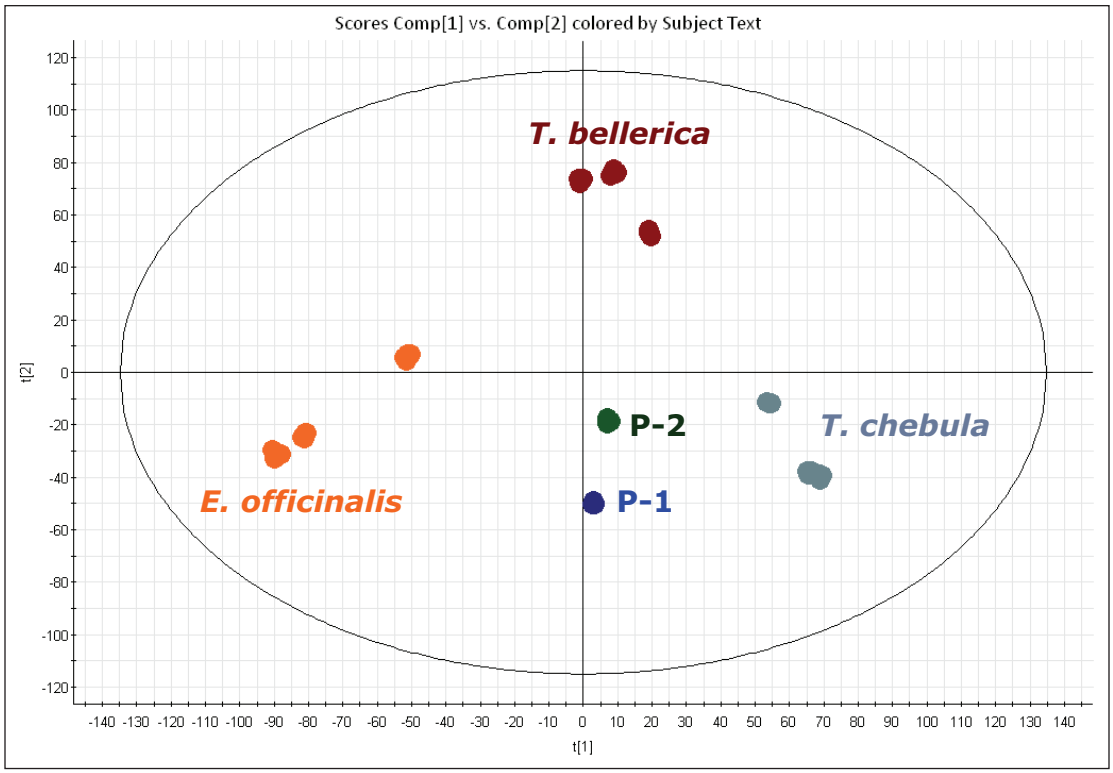

Figure 6. PCA scores plot of the two commercial Triphala products and the three herbs called for by the Triphala formula.

The two products are positioned close to the center of the triangle seemingly formed by the three herbs, *T. bellerica*, *T. chebula*, and *Emblica officinalis*. This suggests that the two commercial products are highly likely to be an equally portioned mix of these three herbs.

In future experiements we plan to prepare a solution by mixing the three herbs in equal portion, and performing the UPLC/oaTOF MS^E/MSA, analysis workflow in the hopes that the PCA scores plot will show a correlation of this mixture with the P-1 and P-2 commercial products.

CONCLUSIONS

- The UPLC/oaTOF MS^E/multivariate statistical analysis workflow offers an effective approach to obtain answers for complex samples in a complicated sample set.
- PCA analysis shows that the chemical content of *Terminalia* differs from species to species. Within the same species, plant location and collection time also affects the chemical content. Chemical identities of key markers can be obtained from PCA loadings plot.
- The two commercial products of triphala appeared to be the results of mixing *T. chebula*, *T. bellerica*, and *E. officinalis*. Further experimental analysis and data mining are required to specifically understand the mixing ratio.

References:

1. Kirtikar KR et al. Indian Medicinal Plants Oriental Enterprises, 2nd edn. Uttranchal, India, 2001, 5, 1415-1439.
2. Ahmad I, Mehmood Z, Mohammad F. Screening of some Indian medicinal plants for their antimicrobial properties. J Ethnopharmacol. 1998, 62:183-193.
3. Chattopadhyay RR and Bhattacharyya SK. *Terminalia* chebula: An update. Pharmacog Rev 2007, 1:151-156.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com