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Differentiation of *Actaea* Species by NMR Metabolomics Analysis

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Abstract

The ^1H NMR spectra of crude extracts from a total of 33 *Actaea* samples were acquired and analyzed for their species- and plant part-specific metabolomic characteristics by identifying fingerprint resonances via visual observation as well as a chemometric approach using principal component analysis (PCA). The main study subjects were the roots/rhizomes and aerial parts of three American species, *Actaea racemosa* (AR), *Actaea podocarpa* (AP) and *Actaea cordifolia* (AC). AP exhibited an already visually distinct chemical profile from those of the other two species. The species-characteristic resonances were identified as analytical chemotaxonomic markers. AR and AC exhibited visually similar ^1H NMR spectral profiles that required statistical analysis for differentiation. Several characteristic peaks and peak patterns were identified for each group of samples. Together with the three American *Actaea* species, the characteristics of the ^1H NMR spectra of Asian species are also discussed. A statistical analysis method using PCA was employed to provide the metabolomic profile for visually minor but analytically significant chemotaxonomic differences. PCA scores allowed differentiation between the three American *Actaea* species, as well as the ability to differentiate between the various plant parts (aboveground vs. roots/rhizomes).

Keywords

Metabolomics; ^1H NMR Fingerprinting; *Actaea racemosa* L.; *Actaea podocarpa* DC.; *Actaea cordifolia* DC.; Cycloartane triterpenes

1. Introduction

Black cohosh (*Actaea racemosa* L., AR) is a plant that has a long and diverse history of medicinal use. Roots and rhizomes of AR were traditionally used by North American indigenous people and early settlers to North America for treatment of a variety of conditions, most notably for indications of premenstrual syndrome (PMS), menstrual pain

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and cramping [1][2]. In contemporary societies, AR is widely available as a form of dietary supplements that contain or are produced with dried plant material. As the resources mostly rely on wild collection, legitimate plant authentication is required. Contamination of AR dietary supplements with other *Actaea* species occurs in the global marketplace for two reasons. First, harvested plant species may be misidentified with other American species, such as *A. podocarpa* DC. (AP) and *A. cordifolia* DC. (AC), because of morphological and habitat similarities. Second, due to economic considerations, high demand and expense, AR can be found to be substituted with less valuable material from other *Actaea* species.

Recently, ^1H NMR-based metabolic fingerprinting using statistical analysis methods has received considerable attention as an indirect approach to authenticate botanicals [3–6]. Representing a metabolomic approach to botanical analysis, it allows for the classification of plants, recognition of adulteration and identification of adulterants, and quality control of botanical products [7]. One major advantage of an NMR-based metabolomic analysis is that it can detect and quantitate unidentified metabolites using estimated or subsequently determined molecular weights.

The authenticity and appropriate quality of raw botanical materials relates to both the proper taxonomy and part of the source plant. While *Actaea* botanicals have historically been limited to use the roots and rhizomes (RR), the components of the aerial parts (PX) represent a potential adulterant. Our prior work on PX constituents of AR led to the elucidation of 12 new triterpenes and two flavones [8]. This represented the first systematic study of ARPX and it revealed the presence of distinct phytochemistry within the aerial parts of AR when considering the >200 compounds identified from ARRR. In contrast, little is known about the metabolomic profile of non-medicinal *Actaea* species such as AP and AC.

For purposes of botanical standardization and botanical safety, however, it is necessary to consider such species as potential adulterants or contaminants, especially as they are known and/or likely to contain distinctly different metabolites. NMR-based metabolomics is a useful methodology for this purpose as it can capture and/or identify both known and unknown constituents. Newly identified characteristic components could potentially serve as chemotaxonomic marker compounds for a given species. Moreover, NMR possesses additional advantages over MS-based methods. First, it can readily analyze the molecular composition, provided that characteristic signals are known, all with relatively simple sample preparation. Secondly, NMR represents a universal and genuinely quantitative detector for all molecules containing NMR-active nuclei (e.g., ^1H), whereas MS shows a more selective detection behavior and requires compound-specific calibration due to inherent variations of ionization efficiencies.

This study identified characteristic fingerprint resonances in the ^1H NMR spectra of the three main American *Actaea* species, including AR, AP and AC, along with some Asian species. Measurements were performed on both aerial parts and roots/rhizomes, and a chemometric approach using principal component analysis (PCA) was applied.

2. Experimental

2.1 Plant Material

A total of 33 samples of different *Actaea* species were used for this study, including three American species: AR, AP, and AC. These three were selected due to the high probability of finding them intermixed during a wild collection of AR, but also because of their shared habitat and morphological similarity. For each of the American species, at least 5 samples from different regions and years were collected in different locations and/or years. Asian *Actaea* species were included for reference purposes only, as the number of available authenticated plant samples was limited, thereby imparting a limit on the statistical analysis. As shown in Table 1, samples denoted BC404, BC581 BC582, BC624, and BC626 were cultivated and collected from the Dorothy Bradley Atkins Medicinal Plant Garden at UIC. Sample BC629 was cultivated in Chillicothe, Ohio, and the specimen was authenticated and deposited in the Herbarium of the Field Museum of Natural History (Chicago, Illinois) by Dr. Doel Soejarto. Other American species were acquired through wild collections in Virginia, North Carolina, and Tennessee by Drs. Daniel Fabricant and Gwynn Ramsey. Voucher specimens are housed at the Searle Herbarium, Field Museum of Natural History and the Ramsey-Freer Herbarium, Lynchburg College (Lynchburg, Virginia). Cultivated Asian species were acquired through Dr. Takahisa Nakane from Showa Pharmaceutical University, Medicinal plant garden (BC587, BC588) and Tokyo Medicinal plant garden (Tokyo, Japan, BC583–586). BC393, and BC395–7 were obtained from PureWorld Botanicals Inc. (current known as Naturex Inc). The collected samples were originally authenticated as *Cimicifuga*. However, the revised naming system was used here since the genus *Cimicifuga* was reclassified to genus *Actaea* in 1998, and many of the species names have changed as a result [9]. The plant part codes that were used in this study are PX for aerial parts and RR for roots/rhizomes in accordance with the established coding system of UIC/NIH Botanical Center Plant.

2.2 Extraction and Sample Preparation

Samples were prepared as described previously [10]. A 10 mL aliquot of 70% aqueous MeOH was added to 1 g of dried, ground plant materials and the mixture sonicated for 30 minutes. The extract was left overnight at room temperature and then filtered. The residue was extracted again with the similar procedures and the two filtrates combined. The combined extract solutions were dried in forced air, to yield the crude extract for metabolomic analysis. A volume of 650 μ L of DMSO- d_6 (Cambridge Isotopes, lot#8L-052, 99.9% D) was added to between 15 and 27 mg of dried extract in an Eppendorf vial, followed by sonication for 5 min. After sonication, the vial was centrifuged and 600 μ L of supernatant were transferred to a 5 mm NMR tube, leaving any insoluble residue in the vial. Three of the major *Actaea* triterpenes (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*- β -D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxide (*syn.* 23-*epi*-26-deoxyactein) (**1**) and (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*R*&*S*)-26-hydroxy-3-*O*- β -D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxide (*syn.* 26*R/S*-actein) (**2/3**) were obtained from USP and dissolved in DMSO- d_6 , then acquiring their NMR data as a reference on the same instrument (600 MHz) under the identical parameters settings.

Identification of these compounds in the extract was performed by visual comparison of spectra of the crude extract and the reference [11][12].

2.3 Fingerprinting and Chemometric Analysis

One dimensional ^1H and homonuclear 2D ^1H NMR experiments (gCOSY) were recorded on a Bruker Avance 600 NMR spectrometer equipped with a TXI cryogenic probe with the sample temperature regulated at 25.0 °C (298.0 K). Acquisition parameters were as follows: acquisition time (aq) = 4 s, relaxation delay (d1) = 60 s, pulse width (p1) = 3 μs (30° flip angle), number of scans (NS) = 64, and dummy scans (DS) = 4. The NMR data were processed in TopSpin (versions 3.0 and 3.2; Bruker, Karlsruhe, Germany), NUTS (Professional version 20100424; Acorn NMR Inc., San Francisco, CA), and Mnova (version 10.0.2; Mestrelab Research S.L., Santiago de Compostela, Spain). Observable characteristics of the ^1H NMR spectra are discussed as part of the fingerprinting of each species and plant part.

Multivariate data analysis of ^1H spectra (Bruker AMIX, version 3.9.7) was carried out to recognize patterns and find discriminating signals that could be used to distinguish between American species. Asian species could not be analyzed with this method due to the limited number of samples. Among multivariate data analyses, the most common unsupervised method is principal component analysis (PCA). PCA is designed to extract and display the systematic variation of a complex data matrix. In brief, PCA is a projection method which reduces the original data to selected principal components in order to describe variation within the data. All raw data (e.g., the integral values as a function of chemical shifts in a ^1H NMR spectrum) of samples are plotted in a K-dimensional space (K = number of variables, e.g., chemical shift bins in ^1H NMR spectra). The analyzed spectra are projected onto the line generated by least square analysis (principal component [PC] line) as dots. The score of each sample is obtained along the first PC line. The next PC can also be calculated by projection onto the line which is orthogonal to the previous principal component line or space. In preparation for PCA, all the ^1H spectra were individually processed with the following parameters: EM (LB 0.3 Hz), zero filling to SI = 512k, from the initial TD of 144k, automatic phasing, and automatic baseline correction, using Bruker Topspin version 3.0. The chemical shifts of all spectra were then referenced to the residual proton signal in DMSO- d_5 at 2.500 ppm. Next, a bucket table was created using Bruker AMIX in the pointwise bucketing method between 0.00 ppm and 1.80 ppm. The rows in the bucket table were scaled to the biggest bucket, and the columns were not scaled. The bucket table was then analyzed by PCA.

3. Results and Discussion

3.1 *Actaea racemosa* L. NMR Fingerprinting

The following observations are based on a ^1H NMR of twelve AR samples, six RR and six PX, as shown in Figure 1. For most of the samples, the B region, which is rich with carbohydrate signals, is the most complex region of the spectra, followed by relatively less signals in the A region, which is rich with triterpene signals, and even smaller signals in the C region, which corresponds to the phenolics.

Although it was observed that the composition of the three major compound classes (A, B, and C regions) of functional groups exhibited variation, the spectral pattern of each plant part appeared to be consistent within each of the spectral regions. The spectral patterns of the A region were carefully examined for the 12 AR extracts (Figure S1). All of the RR samples exhibit distinctive signals at δ_H 1.42 (Ad in Figure S1) and 1.38, which correspond to the Me-27 singlets of compounds **1** and **2/3**, respectively. Owing to a reasonable match of other methyl signals and COSY cross peaks [10], it was shown that all of the ARRR samples contained compounds **1** and **2/3**. The presence and positions of particular methyl signals is a strong indication of the existence of specific compounds [13]. In ARPX, the singlet at 1.42 ppm in **1** was not clearly observed, and suggested that content of **1** is very low or possibly absent. The singlet at 1.38 ppm in **2/3**, which is likely to be attributed to Me-27 of **2/3**, was observed in four of the six PX samples (BC025, 404, 582, 624) (Ab in Figure S1). The presence of **2/3** in these four samples was also supported by the appropriate cross peaks present in the COSY spectrum [10] (Figures. S2A&B). However, **2/3** was not observed in BC026 or BC027 samples. Although a singlet at 1.38 ppm corresponded to Me-27 of **2/3**, it should be noted that other compounds may be likely contributors to its increased intensity, since the observed intensity is significantly higher than that of the other methyl signals associated with just **2/3**. Despite the fact that the corresponding compounds were not identified, other characteristic peak patterns present in 1H NMR spectra were examined. Throughout all of the samples, an acetyl singlet cluster in the sub-region 1.93–2.05 ppm, as indicated by a rectangular (Aa) in Figure S1, was observed. Signals in this sub-region are more crowded in RR than in PX. For PX and RR differentiation, peaks at 1.42 (Ad) and 1.04 ppm (Ae) can be used, because they are insignificant or absent in PX extracts. On the other hand, peaks at 1.38 (Ab) and 1.23 ppm (Ac) tend to be more prominent in the PX extracts.

In the C region of the spectrum (Figure S3), three peaks were consistently observed in the RR samples: a doublet at 6.40 (Ca), a doublet at 6.52 (Cb), and a doublet at 6.75 (Cc) ppm. The doublets (Ca) and (Cb) have a roofing effect to each other suggesting that they are coupled to each other. The COSY spectrum supported the coupling between Ca and Cb as well (Figure S4). The signals of the PX samples, in this region, have more variations between them than that in RR.

3.2 *Actaea podocarpa* DC. NMR Fingerprinting

The 1H NMR spectra of three PX extracts and three RR extracts are shown in Figure 2. The most characteristic pattern was observed in the 1.11–1.16 ppm sub-region (Aa in Figure S5) as “a pair of peaks” when resolution enhancement was applied with routine processing parameters (EM, LB –0.3, GB 0.03) (Figure S5). When a more aggressive resolution enhancement of the spectrum was applied using alternative digitation parameters (GM, LB –3.0, GB 0.3) (Figure S5), it revealed “a pair of peaks”, which was an overlap of two signals, but still needed to be further examined to confirm whether the line separation was associated with a coupling constant (J) or two chemical shifts. To examine this pattern, the 1H NMR of one of the APRR samples, BC008, was measured at two different magnetic field strengths, 9.2 T (400 MHz frequency in hydrogen) and that spectrum was compared to an existing 14.8 T (600 MHz frequency in hydrogen) spectrum. It was shown that the distances between major peaks (δ) are 5.8 Hz at 400 MHz and 8.8 Hz at 600 MHz, which is a 1.5-

fold difference. Since the distance is field strength dependent, the line separation was deduced to be chemical shift related. Strong couplings to these characteristic peaks were not observed in the corresponding COSY experiment (Figure S7), which further serves to confirm that these signals are two independent singlets. A large peak at 1.23 ppm (Ab in Figure S5) was observed in all of the APPX and APRR extracts. In both PX and RR samples, only weak peaks were observed for the region of higher frequency > 0.42 ppm, suggesting that the cycloartane triterpene content of AP is relatively low. In our previously reported qNMR research on cycloartane triterpene content, the different parts of AR were found as **1** to be $2.1 - 3.3\% \pm 0.7\%$ and $1.1 - 4.0\% \pm 1.5\%$ in PX and RR, respectively [10]. Compared to AR and AC extracts, the cycloartane triterpene content of AP is significantly lower. By comparison with the PX and RR extracts, the PX extracts lack the following characteristic signal patterns that were present in the RR samples: most notably, five sharp singlets between 0.56–0.71 ppm (Ac in Figure S5) and those peaks associated with phenolic compounds between 6.0–7.6 ppm (Ca in Figure S5) were observed in all three RR samples.

3.3 *Actaea cordifolia* DC. NMR Fingerprinting

The ^1H NMR spectra of three ACRR extracts and two of the ACPX extracts are shown in Figure 3. As in the spectra of AR and AP, most of the signal intensity was found in the B region (carbohydrate), followed by the A region (triterpenes) and the C region (phenolics). The relative quantities of triterpene content as measured by integration of both PX samples is lower than that of the RR samples.

Some characteristic sub-regions were identified in the ^1H NMR spectra (Figure S8). In all of the spectra including those of PX and RR, several sharp signals were observed in the sub-region 1.93–2.05 ppm (Aa), which is populated principally by acetyl methyl groups. No significant peaks appearing at 1.96 ppm were observed in most of the extracts except for BC015. Since a sharp singlet at 1.96 ppm can be assigned to 12-OAc of the reference compounds **1** and **2/3**, BC015 was checked for compounds **1** and **2/3** with other methyl signals in the ^1H spectrum as well as pertinent cross peaks in the COSY spectrum (Figure S10). Compounds **1** and **2/3** exhibited specific signals at 1.41 and 1.38 ppm and were clearly observed along with another five methyl groups in a crowded region. The COSY spectrum of BC015 showed key cross peaks similar to those of both **1** and **2/3**, such as H-16/H-15a, H-16/H-15b, and H-16/H-17. In contrast, these correlations were not observed in the COSY spectrum of BC016 (Figure S10) as well as that of H-12/H-11ab. Cross peaks from H-1'/H-2' and H-3/2a&b were also observed in BC016, which is consistent, because the 3-*O*-xylopyranoside moiety is not unique to compounds **1** and **2/3**. From examination of both the 1D- ^1H NMR and 2D-COSY, BC015 most likely contains the compounds **1** and **2/3**.

However, other AC samples clearly did not show the presence of **1** and/or **2/3**. According to the literature, reports on compounds from AC are very limited and neither compounds **1** nor **2/3** have not been previously described [14–16]. Further research would be necessary to confirm the presence or absence of **1** and **2/3** in AC.

In terms of differentiation between RR and PX, diagnostic peaks were suggested for each group. A sharp singlet at 1.03 ppm (Ac) is present in all of the RR extracts. The same pattern was observed in ARRR as well, but not in ARPX. The PX samples have two sharp peaks at

1.13 and 1.14 ppm (Ab). The most prominent differences between RX and RR reside in the C region and appears as a cluster of signals between 6.0–6.7 ppm (Ca).

3.4 Summary of NMR fingerprinting of American *Actaea* Species

For the three American species, spectra for 23 samples were acquired and their characteristic peaks were discussed in the previous section. To simplify the comparisons between the six groups of *Actaea* species, a sum of ^1H spectra within each group was assembled using Bruker Topspin software (Figure S11). In this section, the key spectral patterns for the identification of species/parts are summarized. AP samples may be distinguished from both AR and AC by strong marker peaks at 1.11 – 1.16 ppm (Aa). An absence of acetyl groups can also represent a marker for AP. Triterpene patterns of AC and AR are particularly similar. Reference compounds **1** and **2/3** were identified in all of the ARRR extracts, but only in one of the AC extracts. This observation needs separate follow-up for further investigation. A possible chemotaxonomic marker peak at 1.03 ppm (Ab) for distinguishing between RR and PX in AR and AC was identified. Comparing all investigated species, RR appears to have a greater carbohydrate content than PX, as is especially reflected by peaks appearing at 3.24, 3.40 3.55, and 3.57 that are most prominent in the B region of the spectra of RR extracts.

3.5 Asian Species Fingerprinting

In addition to the three American species from Japan, NMR profiles were also obtained for four Asian *Actaea* species, namely: *A. heracleifolia* (AH), *A. biternata* (AB) (Inushoma), *A. japonica* (AJ) (Obashoma), *A. simplex* (AS) (Sarashinashoma). All of these samples predominantly contained the ubiquitous carbohydrate signals, but also present were the corresponding signals for triterpenes and phenolic compounds. The spectra obtained for the Asian species are shown in Figures 4 and S9, together with sum of the spectra from the American species. It is noteworthy that prominent cycloartane methylene signals associated with the C-19 cyclopropanes were observed in all of the Asian samples in the 0–0.62 ppm region of the spectrum. Fingerprint characteristics of each species are not described here, mainly because there was an insufficient number of samples available for each species. However, spectra of the Asian species were carefully examined in order to be able to differentiate them from the American species, especially *A. racemosa*. Most of the samples exhibited a cluster of acetyl methyl signals. Chemotaxonomic marker signals for compounds **1** and **2/3** (1.38 and 1.42 ppm, Aa in Figure S11) were *not* observed for the ^1H spectra of most of the Asian *Actaea* extracts, except in one sample of ASRR (BC587). The COSY spectrum of BC587 (Figure S12) did not show distinguishable cross peaks associated with actein type compounds and the other ASRR (BC393) did not exhibit these marker signals in their ^1H spectra. Although comprehensive NMR data analysis ($^1\text{H}/\text{COSY}$ of BC587 and BC393, Figure S12) failed to detect the presence of **1** and **2/3** in this study, it is possible for ASRR to contain **1** and **2/3**, since they have been isolated from AS previously [17]. The reported yields of **1** and **2/3** from ASRR were 0.08 and 0.01%, respectively. Additional investigations would be necessary to determine the presence of **1** and **2/3** in ASRR. Extracts exhibiting strong signals in the 1.03 ppm (Ab) range tend to be present in RR. The compound giving rise to this characteristic signal has not yet been identified.

3.6 Chemometric Analysis of the ^1H NMR spectra of *Actaea* species

The ^1H NMR spectra of PX and RR extracts of three American *Actaea* species were subjected to a detailed analysis using AMIX NMR Statistics software, with a total of 23 spectra in a bucket table. Pointwise bucketing led to the definition of 7872 buckets for each spectrum between 0–1.8 ppm. This is equivalent to 43 or 44 buckets for every 0.01 ppm-interval of the ^1H NMR spectra. PCA of this extensive bucket table revealed that 95.9 % of the total variance could be explained by eight principal components (PCs). PC1 explained 65.2% of the total variance. Regarding the influence, all of the 23 spectra fitted the model with a probability of 95 %.

A plot of PC1 vs PC 2 was able to explain 76.0 % of the total variance. By looking into 2-dimensional sub-spaces (Figure 5), it becomes evident that the spectra of the same species occupy similar positions (scores) with respect to the corresponding part of the variance. The corresponding variance can be seen in the 2D loadings plot: Both RR and PX of AP spectra are found on the left side of the scores plot. On the 2D loadings plot, it is shown that the buckets that are contributing to the AP cluster were buckets at 1.14, 1.13, and 1.15 ppm, which match with the AP-characteristic fingerprint signals discussed earlier. AC spectra are clustered at the top of the scores plot. While distribution of ARRR overlaps with ACPX BC022 and BC023, all of the ACRR samples appear above the ACPX samples. Specific buckets that allow direct differentiation between AC and AR were not identified, but it is reasonable to conclude that PC2 contributes to their differentiation.

Following PCA, the buckets that include the fingerprinting peaks were conspicuously plotted in the edge of the 2D loadings separated from the crowd of buckets, such as 1.13–1.16 ppm for AP and 1.04 ppm for ARRR and ACRR. Upon visual examination, which is described in sections 3.1, 3.2, and 3.3, it was difficult to account for the numerous but relatively small peaks. However, the PCA method includes all of the buckets of the entire spectra, including prominent fingerprinting peaks. Buckets, which only a statistical method can take into account, are shown as a cluster in the 2D loadings plot and are marked as PCA-only region in Figure 5. In these instances, the outer area of the 2D loadings is a fingerprinting region, and the center of the 2D loadings is the PCA-only region.

Plotting the same datasets with different groupings of PCs (Figure 6, PX samples with dotted and RR with solid lines) allows differentiation between PX and RR with minimal overlap and regardless of the species. PC2 remains a main contributing variant for this differentiation.

As illustrated by this data, PCA analysis of ^1H NMR spectra of crude extracts is a useful metabolomic and phytochemical approach for distinguishing *Actaea* species and/or plant parts. Though this approach does not require identification of phytoconstituents by the observed signals, it can be very useful for suggesting chemotaxonomically significant marker peaks, with the potential for identifying target specific compounds and can serve as marker compounds for ^1H NMR PCA-based quality control of *Actaea* botanical preparations as principal component contributors.

3.7 Summary

The ^1H NMR spectra of crude extracts from a total of 33 *Actaea* plant samples were obtained and analyzed for their species- and plant part- specific metabolomic characteristics by identifying both fingerprint peaks by visual observation and chemometric analysis using PCA. The main study subjects were the roots/rhizomes (RR) and aerial parts (PX) of three American species, *A. racemosa* (AR), *A. podocarpa* (AP) and *A. cordifolia* (AC). Qualitative analysis was carried out and fingerprint peaks were identified. AP exhibited a distinctly different chemical profile and strong chemotaxonomically significant marker peaks were identified for this species. AR and AC were shown to have similar ^1H spectral patterns that are not easily distinguishable without a statistical method. The presence of (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*- β -D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 23-*epi*-26-deoxyactein) (**1**) and (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*R*&*S*)-26-hydroxy-3-*O*- β -D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 26*R/S*-actein) (**2/3**) remain unclear as a diagnostic characteristic for ARRR. In all ARRR extracts, **1** and **2/3** were identified in the extract by comparison with previously isolated compounds as standards. However, these constituents were more likely present in one sample of another species (ACRR BC015). As discussed above, several characteristic signals and peak patterns can be identified for each group of samples.

Following qualitative analysis, a quantitative statistical method using PCA was employed to provide a metabolomics profile for the visually minor but statistically significant differences. PCA scores allowed differentiation between the three American *Actaea* species, as well as the ability to differentiate between PX and RR plant parts.

Overall, this study demonstrated that metabolomic fingerprinting of botanical extracts by 1D ^1H NMR is a useful technique for the identification of the prevalent *Actaea* species and for the distinction between roots/rhizomes (RR) and/or aerial parts (PX) extracts of each given species. Future analysis of additional numbers of samples for each species would further amplify the findings from this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The ^1H Spectra (600 MHz) of 70% Aqueous MeOH Extracts of *A. racemosa* L.
All spectra were scaled to the residual DMSO- d_5 peak.

Fig. 2. The ^1H Spectra (600 MHz) of 70% Aqueous MeOH Extracts of *A. podocarpa* DC. All spectra were scaled to the residual DMSO- d_5 signal.

Fig. 3. The ^1H spectra (600 MHz) of 70% Aqueous MeOH Extracts of *A. cordifolia* DC. All spectra were scaled to the residual DMSO- d_5 resonance.

Fig. 4. Full ^1H NMR Spectra of Asian Species Along with Sum of American Species.
All spectra were scaled to the DMSO- d_5 resonance.

Fig. 5. Scores (left) and 2D loading (right) plots derived from *Actaea* ^1H NMR spectra. Separation into six plant groups is observed in the score plot. Loading plot shows how buckets contributed to the construction of the principal component space. Edge of the space represents fingerprint region, representing typical buckets for each group. The center of the plot is PCA-only region, which is discussed in text.

Fig. 6. Scores Plots of PC1 vs PC2 and PC2 and PC3 derived from *Actaea* ^1H NMR spectra. PX (black dotted line) samples and RR (blue solid line) samples are plotted in PC1-PC2 space and PC2-PC3 spaces. In both spaces, PC2 contributed the most to the differentiation of PX and RR.

Table 1.

List of plant material used for this study.

Genus	Species	Plant Parts ^a	BC number ^b	Collection date	Collection Site
<i>Actaea</i>	<i>racemosa</i>	PX	BC026	06/29/1999	Sevier County, TN. Elevation 3500 ft.
<i>Actaea</i>	<i>racemosa</i>	PX	BC027	06/29/1999	Sevier County, TN. Elevation 3300
<i>Actaea</i>	<i>racemosa</i>	PX	BC624	06/16/2009	Atkins Garden, Cook County, IL
<i>Actaea</i>	<i>racemosa</i>	PX	BC404	10/01/2008	Atkins Garden, Cook County, IL
<i>Actaea</i>	<i>racemosa</i>	PX	BC582	10/07/2009	Atkins Garden, Cook County, IL
<i>Actaea</i>	<i>racemosa</i>	PX	BC025	06/29/1999	Rockbridge County, VA. Elevation 2481 ft.
<i>Actaea</i>	<i>racemosa</i>	RR	BC036	10/16/1999	Elk township, Chester County, PA
<i>Actaea</i>	<i>racemosa</i>	RR	BC007	06/30/1999	Sevier County, TN. Elevation 3300 ft.
<i>Actaea</i>	<i>racemosa</i>	RR	BC094	11/07/2000	Forbes State Forest, Somerset County. PA
<i>Actaea</i>	<i>racemosa</i>	RR	BC629	06/03/2010	Chillicothe, Ross County, OH
<i>Actaea</i>	<i>racemosa</i>	RR	BC626	06/16/2009	Atkins Garden, Cook County, IL
<i>Actaea</i>	<i>racemosa</i>	RR	BC581	10/06/2009	Atkins Garden, Cook County, IL
<i>Actaea</i>	<i>podocarpa</i>	PX	BC029	06/30/1999	Sevier County, TN. Elevation 3300 ft.
<i>Actaea</i>	<i>podocarpa</i>	PX	BC020	08/26/1999	Swain County, NC. Elevation 2875 ft.
<i>Actaea</i>	<i>podocarpa</i>	PX	BC028	06/28/1999	Rockbridge County, VA. Elevation 3150 ft.
<i>Actaea</i>	<i>podocarpa</i>	RR	BC006	06/30/1999	Sevier County, TN. Elevation 3300 ft.
<i>Actaea</i>	<i>podocarpa</i>	RR	BC008	06/30/1999	Swain County, NC. Elevation 2875 ft.
<i>Actaea</i>	<i>podocarpa</i>	RR	BC011	08/26/1999	Sevier County, TN. Elevation 3300 ft.
<i>Actaea</i>	<i>cordifolia</i>	PX	BC022	08/27/1999	Scott County, VA, Elevation 1800 ft.
<i>Actaea</i>	<i>cordifolia</i>	PX	BC023	06/29/1999	Scott County, VA. Elevation 1800 ft.
<i>Actaea</i>	<i>cordifolia</i>	RR	BC015	08/27/1999	Scott County, VA, Elevation 1800 ft.
<i>Actaea</i>	<i>cordifolia</i>	RR	BC003	06/29/1999	Scott County, VA. Elevation 1800 ft.
<i>Actaea</i>	<i>cordifolia</i>	RR	BC016	08/27/1999	Scott County, VA, Elevation 1800 ft.
<i>Actaea</i>	<i>heracleifolia</i>	RR	BC396	n/a	Pure World Botanicals
<i>Actaea</i>	<i>bitermata</i>	PX	BC586	06/2008	Tokyo Medicinal Plant Garden, Japan
<i>Actaea</i>	<i>bitermata</i>	RR	BC585	06/2008	Tokyo Medicinal Plant Garden, Japan
<i>Actaea</i>	<i>bitermata</i>	RR	BC395	n/a	Pure World Botanicals
<i>Actaea</i>	<i>japonica</i>	PX	BC584	06/2008	Tokyo Medicinal Plant Garden, Japan
<i>Actaea</i>	<i>japonica</i>	RR	BC583	06/2008	Tokyo Medicinal Plant Garden, Japan
<i>Actaea</i>	<i>japonica</i>	RR	BC397	n/a	Pure World Botanicals
<i>Actaea</i>	<i>simplex</i>	PX	BC588	06/2008	Showa Pharmaceutical University, Japan
<i>Actaea</i>	<i>simplex</i>	RR	BC587	06/2008	Showa Pharmaceutical University, Japan
<i>Actaea</i>	<i>simplex</i>	RR	BC393	n/a	Pure World Botanicals

^aPlant Parts codes are as follows: PX aerial parts, RR roots/rhizomes.^bThe BC number is a sole number assigned to each collected plant part at UIC/NIH Center for Botanical Dietary Supplements Research, University of Illinois at Chicago.