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Tissue-based metabolite profiling and qualitative comparison of two species of *Achyranthes* roots by use of UHPLC-QTOF MS and laser micro-dissection

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ABSTRACT

Achyranthes bidentata and *Achyranthes aspera* are saponin and steroid rich medicinal plants, used extensively for therapeutic treatments in Traditional Chinese Medicine (TCM) and Ayurveda. *A. bidentata* is reported to be one of the rare and extensively exploited medicinal plant species that face the issue of being endangered. Finding qualitative substitute with identical phyto-constituents contributing to similar composition and pharmacological benefits will help in reducing the burden of exploitation of the natural habitats of such plants. In the present study, a comparative metabolite analysis of the whole drug and specific tissues isolated by laser micro-dissection (LMD) was carried out for both the selected species, by use of ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF MS). The results of the study indicate that the cortex and the medullary ray tissues are rich in their content of steroidal and saponin constituents such as (25S)-inokosterone-20,22-acetonide, ginsenoside Ro, bidentatoside II and achyranthoside B. Metabolite profiling of the whole tissues of both the species indicates presence of identical constituents. Thus, it is inferred that *A. bidentata* and *A. aspera* can be used as qualitative substitutes for each other.

1. Introduction

Plants belonging to genus Achyranthes L. are saponin and steroid rich medicinal plants used for various therapeutic benefits in traditional systems of medicine worldwide [1–4]. *Achyranthes bidentata* Blume and *Achyranthes aspera* Linn. are annual herbs belonging to the family Amaranthaceae [5,6]. *A. bidentate* Blume, commonly known as "Niuxi" in Traditional Chinese Medicine (TCM), is used for its pharmacological activities as an anti-inflammatory, anti-cancer, immunomodulatory and anti-osteoporosis drug [6–12]. *A. aspera* Linn., commonly known as "Apamarga" in Ayurveda, is used for its antifungal, anti-bacterial, anti-inflammatory, anti-fertility, anti-cancer and abortification properties [13–19]. Based on the literature survey of the reports cited above, it is found that these two species have been used in TCM and Ayurveda for two common therapeutic effects *viz*., anti-inflammatory and anti-cancer activities. These plants have been reported for similar activities and thus do pose a possibility of having constituents with similar therapeutic activity or similar chemical class, although they may vary quantitatively in their presence. Thus, in this study we aim to provide scientific substantiation on the possibilities of their qualitative substitution by identifying and comparing their phytoconstituents, using microscopic and hyphenated chromatography techniques. There have been no studies carried out till date, for qualitative tissue specific metabolite analysis of *A. bidentata* and *A. aspera* to identify the possibilities of their substitution with each other.

Literature survey reveals that there are no synthetic drug formulations available for active constituents extracted from roots of *A. bidentata* and *A. aspera.* The drug preparation methods in both TCM and Ayurveda differ considerably. In TCM the roots of *A.bidentata* are collected in winter, separated from rootlets, sundried until wrinkled externally, and cut evenly for use as medicine. To prepare processed drug, the dried roots are stir baked in wine [20]. In Ayurveda, the roots of *A. aspera* are prepared into a formulation called *kshara*. In this formulation the water soluble ashes of the roots are used to obtain alkaline substances [21]. Thus, this study envisages to provide insights into the common actives that may be responsible for common therapeutic effect, although different preparation methods are used traditionally. This study provides inferences that may be employed in designing common preparation techniques for formulations from these plants.

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A. aspera is used in Ayurveda for treatment of kidney disorders by taking the dried powder of the roots, with a dose of about 5 g two times a day. For treatment of pain/swelling, the powdered roots are dipped in hot water and the resulting extract is applied on the inflamed regions [22]. Its roots are used in the form of ointments to treat warts and as a tooth brush for its effective action against dental ache and plaque [23–25]. The roots of this plant are also reported to be used in treatment of epilepsy, and its effect is known due to its saponins being able to facilitate the GABAergic neurotransmission [26]. In several studies, the anti-inflammatory and anticancer properties of *A. aspera* are attributed to the presence of steroidal saponins, phenolic acids, alkaloids, tannins, terpenoids and glycosides [27,28]. The roots of *A. bidentata* processed in wine are taken internally for the treatment of dysuresia, nourishment of kidneys and liver, pruritic urticaria, leprosy, aptha and malaria. The juice of the roots is used for treatment of pharyngitis, wounds and eye ailments. The aqueous decoction of the roots is used in the treatment of stranguria, mazischesis and wounds [28]. In reports published till date for both these species and the whole genera of Achyranthes, it is suggested that the fructans and saponins have immunomodulatory effect due to their ability to selectively boost the immunological responses, increase the Th1 cytokine secretions and decrease the Th2 cytokine secretion [29,30]. The anti-cancer activity of these plants has been demonstrated in various in-vitro and in-vivo models and it is suggested that this property can be attributed to the triterpenoid saponins and polysaccharides. These compounds inhibit tumour growth by activation of the immune system and induction of apoptosis in cancerous cells [9,31].

A recent publication by Tyler et al. [32] states that *A. bidentata* is one of those plants in TCM which have a great trade value in the herbal drugmarket in the United States of America, and is vulnerable of being endangered. The National Medicinal Plants Board, New Delhi, India, and Foundation for Revitalisation of Local Health Traditions, Bangalore, India, published a report which states that *A. aspera* is one of the 46 important Indian medicinal plant species that are sourced from the wastelands [33]. These species may not warrant immediate attention for the threat of being endangered, but they do demand implementation of actions to enhance their cultivation outputs and strict quality control standards to avoid adulteration.

There is a rising need to identify qualitative substitutes of plants with such varied and beneficial therapeutic effects and whose natural habitats are being exploited for meeting the needs of herbal drug market. Tissue specific metabolite analysis can help identify constituent rich parts of the plant that can be effectively used for enrichment of herbal drugs or health supplements. There have been no reports published to identify secondary metabolites in specific tissues of roots of these two selected Achyranthes species and provide their qualitative comparison. The UV laser dissection feature of laser macro-dissection (LMD) instrument was used to observe and dissect tissues for specific tissue metabolite analysis. Achyranthes species are well known for their pharmacological activities attributed to the presence of oleanolic acid glycosides (saponins), dammarane saponins, steroids, anthraquinones, flavonoids and phenol carboxylic acids. Based on the literature survey of various mass banks, databases, and scientific publications, we prepared a database of 95 constituents reported in Achyranthes species [34–38]. The extraction solvent used for the present analysis was methanol, as its polarity was close to the solvent used for extraction of the roots in traditional systems of medicine, i.e. water. This solvent selection was suitable for liquid chromatography–mass spectrometry (LC–MS) analysis and could extract most of the constituents that could be extracted with water as a solvent. The present study is the first report that provides such a comparative approach and identification of tissue specific metabolites in these two species.

2. Materials and methods

2.1. Chemicals

Acetonitrile (HPLC grade) used for LC–MS analysis was obtained from E. Merck (Darmstadt, Germany). Other mobile phase modifiers and reagents including formic acid (HPLC grade, purity of 96.0%) were purchased from Tedia Company Inc (U.S.A.). Ultra-pure water used for plant sample treatment and for different experimental steps was obtained from in house installed Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

2.2. Plant materials

Three sample sets of each of the dried roots of *A. aspera* Linn. and *A. bidentata* Blume were collected from commercial herbal drug markets in the state of Maharashtra, India, and herbal drug stores from various regions of China. The authentication of samples was carried out by Professor Zhongzhen Zhao and the voucher specimens were deposited for records in the Chinese Medicines Centre of Hong Kong Baptist University, Hong Kong, China.

2.3. Preparation of microscopy sections

The dissection of microscopic tissues from roots of both the species of *Achyranthes* samples was carried out as detailed by Jaiswal et al. [39,40]. Prior to laser microdissection, the root samples were conditioned by wrapping in non-cellulose paper soaked with ultrapure water. The soaked samples were then kept under vacuum at 25 inHg pressure for a period of 24 h, at room temperature. After softening, the roots were cut into small pieces of about 1–1.5 cm heights and embedded in cryogel matrix (Leica Microsystems, Germany) for cryo-sectioning. Sections of roots with thickness of about 20–25 μm were cut with a cryotome equipment and carefully placed on metallic PET slides, precooled at −15 °C.

2.4. LMD of root tissues

Cryo-sectioning of roots and LMD of specific root tissues were carried out at − 15 °C, by using a Thermo Shandon As620 Cryotome, UK and Leica LMD 7000 system (Leica, Benshein, Germany), respectively. By using the Leica LMD-BGR fluorescence filter system and various magnifications (6.3× and 10×) for tissue inspection, the target tissues were dissected and collected in Eppendorf tube caps prefixed in LMD collecting device. The total area dissected for each specific tissue was about 1×10^6 μm^2. The conditions used for the LMD instrument during dissection were aperture size of 1, exposure time of 115.9 ms, speed value 3 and 2.3× gain.

2.5. Extraction of LMD dissected tissue and the whole root sections

The laser dissected root tissues were centrifuged at 15,000 rpm for 10 min by a Centrifuge 5415R (Eppendorf, Hamburg, Germany) to reduce sample loss and ensure effective collection of tissues. After centrifugation, 100 μL of HPLC grade methanol was added into each Eppendorf tube, and the extraction process was facilitated by sonication of the tubes for 30 min using CREST 1875HTAG ultrasonic processor, USA. After 30 min of sonication, centrifugation of the tubes was carried out at 15,000 rpm for 10 min. The resultant supernatant extracts were collected in HPLC glass vials containing glass inserts with bottom springs. The samples were kept refrigerated at 4 °C until analysis.

The whole root sections were extracted by collecting 5–6 sections of the cryo-dissected samples in Eppendorf tubes. These sections were then extracted with 500 µL of HPLC grade methanol with the procedure identical to the one described for specific laser dissected tissues described above for isolated tissues.

2.6. UHPLC-QTOF MS analysis

The ultra-performance liquid chromatography (UPLC) analysis of samples was carried out by use of an Agilent 6540 accurate – mass Q-TOF LC/MS (Agilent Technologies, USA) system. Separation was carried out by use of a UPLC C_{18} analytical column (2.1 mm × 100 mm, I.D. 1.7 µm, ACQUITY UPLC® BEH, Waters, U.S.A.) equipped with a C_{18} pre-column. The LC–MS analysis was performed at room temperature of 20 °C. The optimised chromatographic conditions used for MS analysis were as follows: dry gas (N_2) flow rate 6 L/min, Vcap 4500, dry gas temperature 300 °C, nozzle voltage of 500 V, fragmentor voltage 150 V, and nebulizer pressure 40 psi. To obtain the identification of the largest number of phytoconstituents and the most appropriate mode of acquisition, the mass spectra acquisition was carried out in both negative and positive modes. Mass scanning range for mass to charge ratio (*m/z*) selected for analysis was 110–1700. The solvent system used for the chromatographic analysis comprised a mixture of water (A) and acetonitrile (B), both containing 0.1% formic acid. The linear gradient conditions were as follows: 0–8 min, 2%–15% B; 8–18 min, 15%–55% B; 18–23 min, 55%–100% B; 23–26 min, 100% B; 26–26.1 min, 100%–2% B, 26.1–30 min, 2% B, flow rate 0.4 mL/min. The injection volume used for each sample was 2.0 µL. Accurate mass calibration was done by using ESI-low concentration tuning mix solution (Agilent technologies, USA) with accuracy error threshold of 10 ppm.

2.7. Data analysis

Analysis of data obtained from LC–MS was carried out with Agilent Mass Hunter Workstation Software-Qualitative Analysis (version B 4.00, Build 4.0.479.5, Service Pack 3, Agilent Technologies, Inc. 2011). The following settings were applied during data analysis: extraction restricted retention time of 1.0–30.0 min, charge state of + 1 and − 1 used for positive and negative modes, respectively. The peaks with height ≥ 2000 counts were used for analysis. The elements of C, H, O, and N from 3 to 60, 0 to 120, 0 to 30 and 0 to 30, respectively, were used to generate formulae. The compound absolute height used was ≥ 5000 counts and relative height was ≥ 2.5%. Peak spacing tolerance used for analysis was 0.0025 *m/z* plus 7.0 ppm. The peak lists from the data analysis with Mass Hunter Workstation software were prepared in Microsoft Excel software (Microsoft, Redmond, WA). The results of analyses are depicted with base peak chromatograms (BPCs, with *m/z* range 150–950).

3. Results and discussion

3.1. Macroscopic and microscopic studies of the roots of A. bidentata and A. aspera

The roots of *A. bidentata* were cylindrical in shape, with frequent twisted regions and alternate striations (Fig. 1A). Striations on the outer area of the root depicted the remnants of the rootlets. The roots were about 1.0–1.2 cm in width with some roots also appearing as broad as 1.5 cm. They appeared light brown in dried conditions. *A. aspera* roots appeared less parenchymatous and had fewer striations and rootlet remnants compared to *A. bidentata* roots. *A. aspera* roots were about 0.8–1.0 cm in thickness and like *A. bidentata* had occasionally twisted regions and wrinkles along the outer region of the roots (Fig. 1B). The inner core of the roots appeared parenchymatous and fleshy around the cortex region for both the species.

Fig. 1. Pictorial representation of dried roots of (A) *Achyranthes bidentate* Blume and (B) *Achyranthes aspera* Linn.

In Figs. 2 and 3, typical microscopic features of *A. bidentata* and *A. aspera* root sections are depicted under white light and blue fluorescence light, respectively. The roots of *A. bidentata* exhibited a good development of tertiary structures formed by differentiation of cambium. Medullary rays and parenchyma cells separated the tertiary vascular bundles, and the tertiary vascular bundles were found more in number towards the centre. The outer peripheral region showed the presence of a thin indistinct epidermis, cortex and protoxylem. The secondary structures found were the medullary rays and the metaxylem interspersed with parenchyma cells.

In case of *A. aspera*, the primary and secondary tissue structures were common to those of *A. bidentata*. Difference was found in the placement of the tertiary vascular bundles. The tertiary vascular bundles were arranged radially along the centre of the roots. The parenchyma cells were not found to be as dense as observed in *A. bidentata* towards the central region. Under blue fluorescence, the cortex cells exhibited bright fluorescence and the cork appeared dark reddish brown in colour. The tertiary vascular bundle cells exhibited a brighter fluorescence compared to the medullary rays and were prominent and easy to distinguish.

3.2. Characterization of metabolites

The constituents identified are listed in Table 1, with the calculated and observed *m/z* values, molecular formulae, retention time (Rt), and identified molecular species. Based on the reported *m/z* values and the observed *m/z* values, tentative identification of the constituents was carried out. The constituents identified in both positive and negative modes are listed in Table 1. However, negative mode was found to identify the most constituents present in roots of both selected plant species, with good peak resolution.

3.3. Identification of metabolites by UHPLC-QTOF MS

Representative BPCs of whole root tissues of both the species, the cryogel used as control and methanol used as solvent blank are indicated in Fig. 4. A database of 95 constituents was prepared and used for identification, of which 26 constituents were identified in the species studied. The database is provided for reference (Supplementary material). The cryogel used for cryo-sectioning contains glycols and polyvinyl alcohols which exhibited distinct peaks between 12 and 13 min retention time. Methanol used as solvent eluted few minor peaks after 18 min retention time, and did not show any interference with the identification of constituents. Negative mode resulted in identification of greater number of compounds with better resolution and chromatograms of negative mode are indicated in Fig. 4.

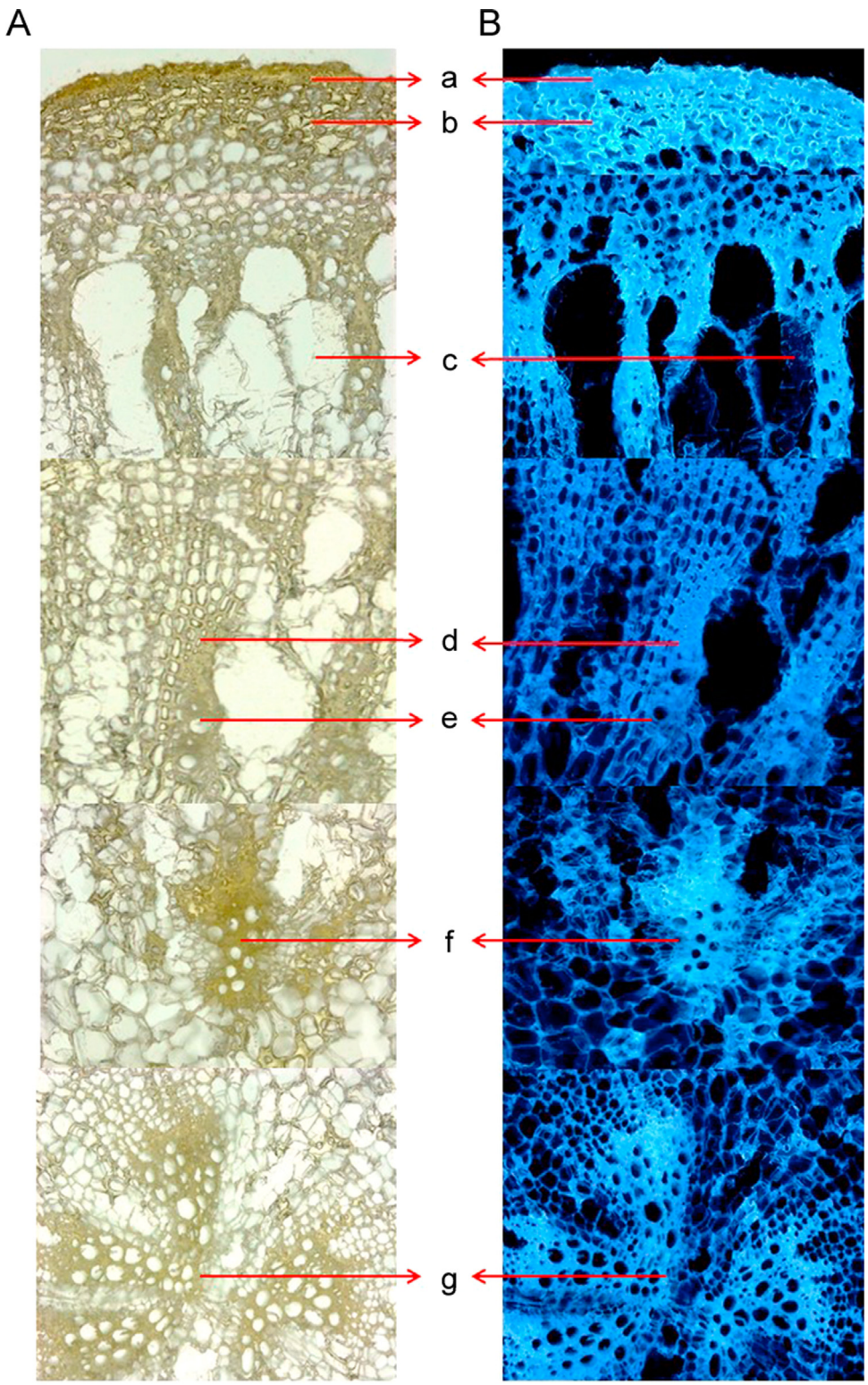

Fig. 2. Microscopic characteristics of *A. bidentata* under (A) white light and (B) blue fluorescence. The various tissues denoted are: (a) cork, (b) cortex, (c) protoxylem, (d) medullary rays, (e) metaxylem, and (f and g) tertiary vascular bundles.

As observed in Fig. 4, several constituents were found in common in both *A. bidentata* and *A. aspera*. Out of the 26 identified constituents, 23 constituents were found to be common between root samples of both the species. In *A. aspera,* 1-Tritriacontanol, 11-methyl- and chikusetsu saponin-IVa butyl ester were exclusively found. Palmitic acid was found to be present only in the *A. bidentata* species. The identified group of constituents can be classified into different classes, as listed in Table 2.

The constituents, glutamic acid, niacin, achyranthine, 5-hydroxymethyl-furfural, myristic acid, behenic acid and 1-Tritriacontanol, 11-methyl-, were detectable only in the positive mode and hence are not represented in Figs. 4–6. These components are important

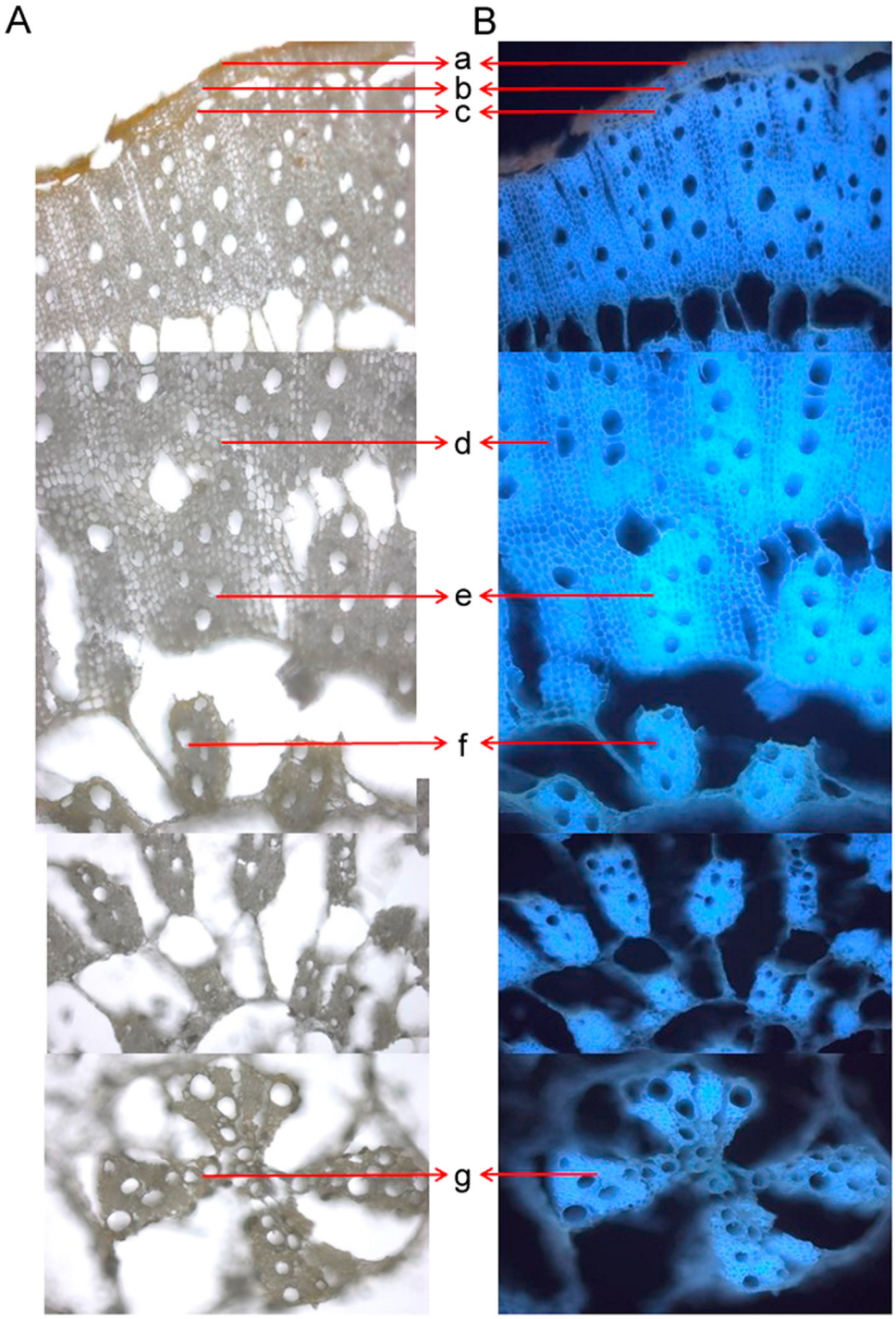

Fig. 3. Microscopic characteristics of *A. aspera* under (A) white light and (B) blue fluorescence. The various tissues denoted are: (a) cork, (b) cortex, (c) protoxylem, (d) medullary rays, (e) metaxylem, and (f and g) tertiary vascular bundles.

constituents of the plant as they include vitamins and fatty acids which contribute to other activities of the plants such as anti-oxidant effect. However, the most prominent and well-known constituents of the plant are its saponins and steroids detected in the negative mode. Overall, both *A. bidentata* and *A. aspera* species have comparable number and most of their constituents are identical. Based on these findings we suggest that these species can be substituted for each other for specific therapeutic properties, which include their anti-inflammatory and anti-cancer properties that are attributed in literature due to the presence of saponins and steroids. In Fig. 4, the constituents eluted in the whole extract of both the species are elucidated. In Figs. 5 and 6, the tissue specific constituents that were identified are elucidated. These identifications provide information of the presence of constituents in specific tissues. This information can be used for further exploration by herbal drug formulators, herbal drug researchers and biotechnologists to enhance or modify the extraction of constituents from these tissues and prepared constituent-enriched products.

A tentative quantitation of the constituents can be carried out for the identified constituents based on the peak heights of the constituents, as the experimental conditions were the same for both the samples. It was observed that in *A.bidentata*, the

Table 1
List of constituents identified in *Achyranthes aspera* and *Achyranthes bidentata* species.

Peak no.	Compound name	Molecular formula	Calculated mass values	Rt (min)	Species	Calculated m/z values	Observed m/z values	Δ ppm	Samples[a]							
									CW	CA	CB	CC	IW	IA	IB	IC
1.	Glutamic acid	$C_5H_9NO_4$	147.0533	0.842	$(M+H)^+$	148.0604	148.0604	1.05	+	+	+	+	+	+	+	+
2.	Niacin	$C_6H_5NO_2$	123.0327	1.124	$(M+H)^+$	124.0393	124.0394	5.26	+	+	+	+	+	–	–	–
3.	Achyranthine	$C_6H_{11}NO_2$	129.0779	1.196	$(M+H)^+$	130.0863	130.0863	8.29	+	+	+	+	+	–	–	–
4.	Ascorbic acid	$C_6H_8O_6$	176.0331	1.422	$(M-H)^-$	175.0248	175.0244	−5.54	+	+	+	+	+	–	–	–
5.	5-hydroxymethyl-furfural	$C_6H_6O_3$	126.0323	4.488	$(M+H)^+$	127.0390	127.0388	4.51	+	–	–	–	–	–	–	–
6.	20,26-dihydroxyecdysone	$C_{27}H_{44}O_8$	496.3042	6.555	$(M-H)^-$	495.2963	495.2972	2.09	+	+	+	–	+	–	+	+
7.	Beta-D-Fructofuranoside	$C_{10}H_{20}O_6$	236.1254	7.552	$(M-H)-[-H_2O]$	217.1081	217.1074	−5.35	+	+	+	+	+	–	+	+
8.	Beta-ecdysone	$C_{27}H_{44}O_7$	480.3105	7.973	$(M-H)^-$	479.3014	479.3019	−1.89	+	–	–	–	+	–	–	–
9.	Cis-N-Feruloyltyramine	$C_{18}H_{19}NO_4$	313.1316	8.201	$(M+HCOO)^-$	358.1296	358.1305	−2.52	+	–	–	–	–	–	–	–
10.	Chikusetsu saponin-IVa butyl ester	$C_{46}H_{74}O_{14}$	850.5058	10.712	$(M+CH_3COO)^-$	909.5217	909.5200	2.39	–	–	–	–	+	+	+	+
11.	Stachysterone D	$C_{27}H_{42}O_6$	462.2993	11.548	$(M-H)^-$	461.2909	461.2911	−2.58	+	–	–	–	+	–	+	+
12.	(25S)-20,22-O-(R-ethylidene)inokosterone	$C_{29}H_{46}O_7$	506.3258	11.848	$(M-H)^-$	505.3171	505.3139	6.34	+	–	–	–	+	–	–	–
13.	(25S)-inokosterone-20,22-acetonide	$C_{28}H_{44}O_7$	492.3103	11.930	$(M+CH_3COO)^-$	551.3226	551.3237	9.74	+	+	–	–	+	+	+	+
14.	Zingibroside R1	$C_{47}H_{80}O_{18}$	991.5486	12.186	$(M+CH_3COO)^-$	991.5483	991.5486	−0.7	+	+	–	–	+	+	+	+
15.	Achyranthoside D	$C_{53}H_{82}O_{25}$	1118.5161	13.565	$(M-H)^-$	1117.5072	1117.5091	−0.12	+	–	–	–	+	–	–	–
16.	Ginsenoside Ro	$C_{48}H_{76}O_{19}$	956.4966	13.665	$(M-H)^-$	955.4908	955.4896	4.32	+	+	+	+	+	+	+	+
17.	Sulphachyranthoside D	$C_{53}H_{82}O_{28}S$	1198.4715	13.953	$(M-H)^-$	1197.4641	1197.4648	−0.48	+	+	+	+	+	+	+	+
18.	Bidentatoside	$C_{47}H_{70}O_{20}$	954.45	14.070	$(M-H)^-$	953.4388	953.4425	6.17	+	+	+	+	+	–	–	–
19.	Chikusetsu saponin IVa	$C_{42}H_{66}O_{14}$	794.4459	14.207	$(M-H)^-$	793.4380	793.4388	−1.23	+	+	+	+	+	+	+	–
20.	Bidentatoside II	$C_{40}H_{62}O_{13}$	750.4144	14.409	$(M+HCOO)^-$	795.4172	795.4176	−0.72	+	+	+	+	+	+	+	+
21.	Achyranthoside B	$C_{47}H_{72}O_{20}$	956.4604	15.155	$(M-H)^-$	955.4544	955.4533	2.01	+	+	+	+	+	+	+	+
22.	Momordin Ib	$C_{36}H_{56}O_9$	632.3933	16.138	$(M-H)^-$	631.3852	631.3861	−1.64	+	+	+	+	+	+	+	+
23.	Palmitic acid	$C_{16}H_{32}O_2$	256.2414	16.475	$(M+CH_3COO)^-$	315.2541	315.2554	−4.05	+	+	+	+	–	–	–	–
24.	Myristic acid	$C_{14}H_{28}O_2$	228.2089	16.855	$(M+H)+[-H_2O]$	211.2056	211.2042	0.23	+	+	–	–	+	+	+	+
25.	Behenic acid	$C_{22}H_{44}O_2$	340.3345	17.530	$(M+H)+[-H_2O]$	323.3308	323.3388	−1.07	+	–	–	–	+	+	–	–
26.	1-Tritriacontanol,11-methyl-	$C_{34}H_{70}O$	494.542	18.510	$(M+NH_4)+[-H_2O]$	494.5659	494.5649	1.43	–	–	–	–	+	–	+	–

[a] It indicates the presence or absence of specific phytoconstituents in respective samples. Presence of the phytoconstituent is denoted by (+) and the absence is denoted by (-).

Table 2
Classification of constituents identified in *A. bidentata* and *A. aspera* in the developed LC–MS method.

Classes of constituents	Constituents identified in the analysis
Saponins (dammarane saponins)	Chikusetsu saponin-IVa butyl ester, achyranthoside D, ginsenoside Ro, sulphachyranthoside D, chikusetsu saponin IVa, and achyranthoside B
Saponins (oleanolic acid glycosides)	Zingibroside R1, bidentatoside, bidentatoside II, momordin Ib
Steroids	Beta-ecdysone, 20,26-dihydroxyecdysone, stachysterone D, (25S)-20,22-O-(Rethylidene) inokosterone, and (25S)-inokosterone-20,22-acetonide
Glycosides	Beta-D-Fructofuranoside
Flavonoids and carboxylic acids	Glutamic acid and achyranthine
Other compounds	Niacin, ascorbic acid, 5-hydroxymethyl-furfural, cis-N-Feruloyltyramine, palmitic acid, myristic acid, 1-Tritriacontanol,11-methyl-, behenic acid

ginsenoside Ro, sulphachyranthoside D, and bidentatoside had peak heights about 4 times higher than in *A. aspera*. Ascorbic acid indicated peak height 3 times higher, and (25S)-inokosterone-20,22-acetonide exhibited peak height twice higher in *A. bidentata* than in *A. aspera*. This is only the tentative estimation of the quantitative differences between these species, as the article focuses mainly upon qualitative differences between these two species, based on the identification of various constituents.

3.4. Tissue-specific metabolite analysis by UHPLC-QTOF MS

For tissue-specific metabolite analysis, three tissues were selected for LMD from root samples of both the species. The cortex, the medullary rays and the tertiary vascular bundles were dissected from each of the three root samples of both the species. Representative BPCs for each of the tissues of *A. bidentata* and *A. aspera* are shown in Figs. 5 and 6, respectively.

The secondary metabolites belonging to the class dammarane saponins that include ginsenoside Ro, sulphachyranthoside D, achyranthoside B, and oleanolic acid glycoside saponins that include bidentatoside II and momordin Ib were found in all three isolated tissues of both *A. bidentata* and *A. aspera*. In *A. bidentata*, in addition to the above-mentioned constituents, the common constituents between the cortex, medullary rays and the tertiary vascular bundles were ascorbic acid and palmitic acid. The cortex and medullary ray tissues exhibited the presence of the steroid 20,26-dihydroxyecdysone, whereas the tertiary vascular bundles did not show the presence of it. The oleanolic acid glycoside saponin zingibroside R1 and the steroid (25S)-inokosterone-20,22-acetonide were found in the cortex, the medullary ray, and the tertiary vascular bundles of *A.*

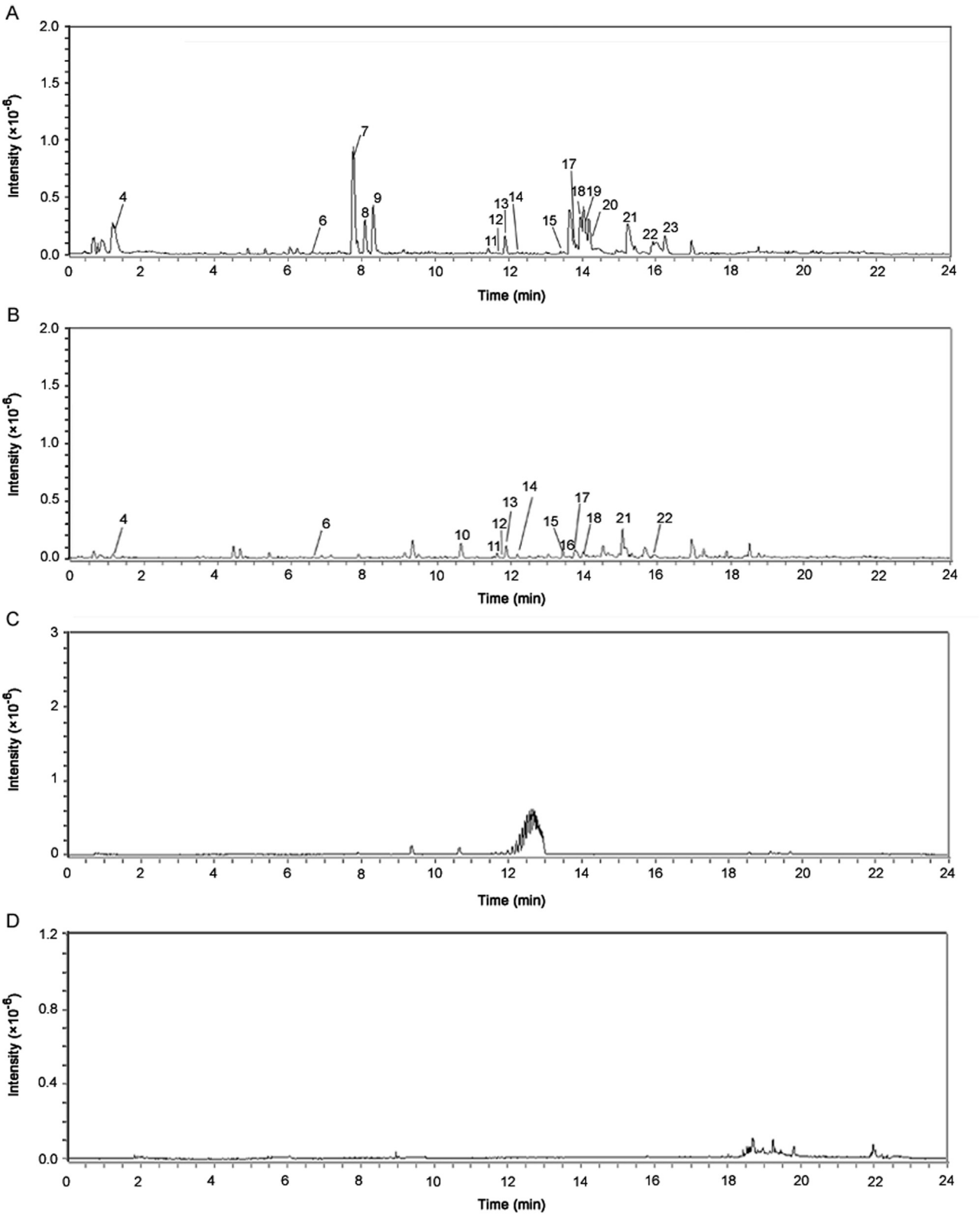

Fig. 4. LC–MS base peak chromatograms of (A) whole tissue sections of *A. bidentata*, (B) whole tissue sections of *A. aspera*, (C) cryogel used as control, and (D) methanol used as solvent blank in negative mode.

aspera and the cortex tissue of *A. bidentata*. The cortex of *A. aspera* did not show the presence of the steroidal constituent, 20,26-dihydroxyecdysone. Based on the tissue specific metabolite analysis results, we infer that the cortex and medullary rays have higher number of dammarane and oleanolic acid glycoside saponins and steroids. Thus, the outer regions of the roots comprising the cortex and the medullary rays can be extruded and used exclusively for enrichment of herbal products for saponin and steroidal content.

A. bidentata and *A. aspera* are both widely used for the therapeutic benefits discussed above in TCM and Ayurveda, respectively.

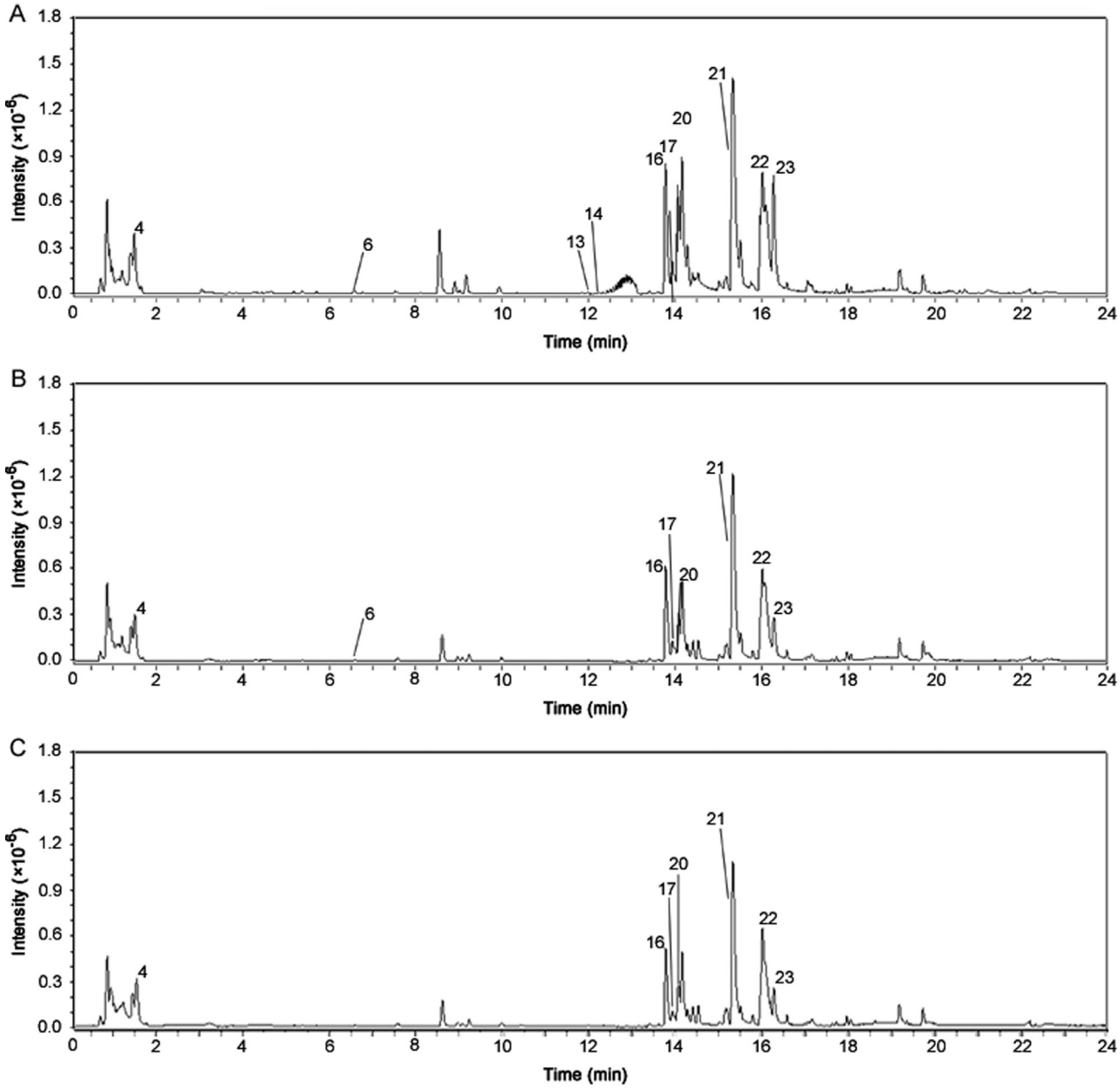

Fig. 5. LC–MS base peak chromatograms of various laser dissected tissues of *A. bidentata* analysed in negative mode. (A) cortex, (B) medullary rays, and (C) tertiary vascular bundles.

A. bidentata is reported to be one of the most extensively used medicinal plants in TCM that face the problem of being endangered due to exploitation of their natural habitats, to meet commercial demands for their medicinal products [32]. In-vitro propagation of plants as an alternative to supplement for the demands of endangered species is a promising discipline. However, it faces the concern of being a time-consuming approach. The reduced adaptability of the plants grown through in-vitro propagation to the natural habitats is a major factor for commercial cultivation [41,42]. Thus, identification of alternate species of the same genus and family, which have the same phytoconstituents, will be of significant help to provide qualitative substitute species for therapeutically beneficial but endangered medicinal plants [43,44]. Identification of substitute species will help herbal drug industrialists, researchers in the field of phyto-chemistry and pharmacology, traditional medicine practitioners and agriculturists to utilise and further investigate the applications of substitute species in traditional medicine and for commercial production of herbal products.

4. Conclusion

From this comparative study, we infer that in *A. bidentata* and *A. aspera* the cortex and the medullary rays are the tissues rich in saponin and steroidal contents and can be used for enrichment of herbal products. The roots of these two selected plant species can be used as qualitative substitutes for each other in traditional medicine practices and for commercial herbal drug production.

Conflicts of interest

The authors declare that there are no conflicts of interest.

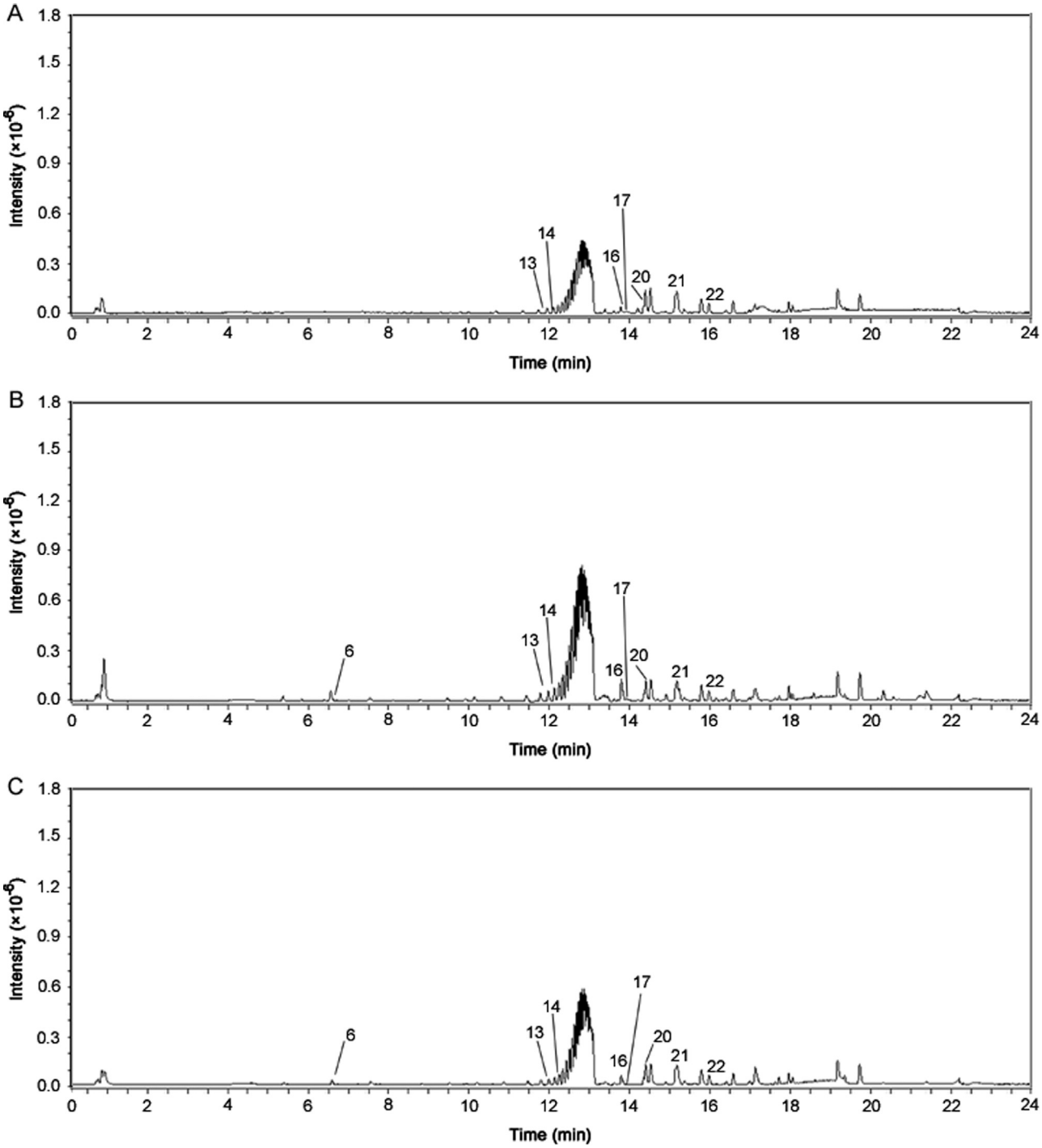

Fig. 6. LC–MS base peak chromatograms of various laser dissected tissues of *A. aspera* analysed in negative mode. (A) cortex, (B) medullary rays, and (C) tertiary vascular bundles.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2017.06.006.

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