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Phytochemical Analysis

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Determination and Comparative Analysis of Major Iridoids in Different Parts and Cultivation Sources of *Morinda citrifolia*

Shixin Deng,* Brett J. West, 'Afa K. Palu and C. Jarakae Jensen

ABSTRACT:

Introduction – Noni is a medicinal plant with a long history of use as a folk remedy in many tropical areas, and is attracting more attention worldwide. A comprehensive study on the major phytochemicals in different plant parts (fruit, leaf, seed, root and flower) and sources is of great value for fully understanding their diverse medicinal benefits.

Objective – To quantitatively determine the major iridoid components in different parts of noni plants, and compare iridoids in noni fruits collected from different tropical areas worldwide.

Methodology – The optimal chromatographic conditions were achieved on a C_{18} column with gradient elution using 0.1% formic acid aqueous formic acid and acetonitrile at 235 nm. The selective HPLC method was validated for precision, linearity, limit of detection, limit of quantitation and accuracy.

Results – Deacetylasperulosidic acid (DAA) was found to be the major iridoid in noni fruit. In order of predominance, DAA concentrations in different parts of the noni plant were dried noni fruit > fruit juice > seed > flower > leaf > root. The order of predominance for asperulosidic acid (AA) concentration was dried noni fruit > leaf > flower > root > fruit juice > seed. DAA and AA contents of methanolic extracts of noni fruits collected from different tropical regions were 13.8–42.9 and 0.7–8.9 mg/g, respectively, with French Polynesia containing the highest total iridoids and the Dominican Republic containing the lowest.

Conclusion – Iridoids DAA and AA are found to be present in leaf, root, seed and flower of noni plants, and were identified as the major components in noni fruit. Given the great variation of iridoid contents in noni fruit grown in different tropical areas worldwide, geographical factors appear to have significant effects on fruit composition. The iridoids in noni fruit were stable at the temperatures used during pasteurisation and, therefore, may be useful marker compounds for identity and quality testing of commercial noni products. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: noni; *Morinda citrifolia*; iridoid; HPLC-PDA

Introduction

Noni (*Morinda citrifolia* Linn.) is a popular medicinal plant indigenous to a wide range of tropical areas, such as southern Asia, the Caribbean, and the Pacific Islands. In addition to the well-known fruit, the leaf, seed, root, and flower of noni plant have also been used traditionally to treat many health ailments, including arthritis, infections, cancers, diabetes, inflammation, pains, among others (McClatchey, 2002; Wang *et al*., 2002). The root was used as a cathartic and febrifuge, and applied externally to relieving pain. The leaf was thought to help wound and ulcer healing, with the fruit being effective for throat complaints, bruises, boils, and wounds. (Anonymous, 1962).

In the past decade, many scientific studies have been conducted on chemical constituents of noni. The noni plant has been found to contain amino acids, anthraquinones, fatty acids, flavonoids, iridoids, lignans, polysaccharides, sterols, sugars and terpenoids (Deng *et al*., 2007; Pawlus and Kinghorn, 2007; Potterat and Hamburger, 2007). Although different chemical profiles have been observed for noni fruit, leaf, root, seed and flower, these plant parts may have some phytochemicals in common, such as the ubiquitous flavonoids. Additionally, iridoids have been identified from noni fruit, leaf and root, and seem to be characterisitc phytochemicals in different noni plant parts (Kamiya *et al*., 2005, 2008; Sang *et al*., 2001; Potterat *et al*., 2007; Samoylenko *et al*., 2006). As a significant taxonomical biomarker for Rubiaceous plants (Inouye *et al*., 1988), iridoids are usually present as glycosides (Dinda *et al*., 2007a). Iridoids or iridoid-rich plants have demonstrated a broad range of biological activities in *in vitro*, *in vivo* and in clinical studies. These include anti-arthritic, anti-inflammatory, antibacterial, antifungal, anticancer, anticoagulant, antioxidant, antivirus, antispasmodic, immunomodulatory, wound-healing and neuroprotective activities (Dinda *et al*., 2007b).

Currently, there are some questions regarding the content and quality of the growing number of commercial noni products (West *et al*., 2006; Palu *et al*., 2005). As such, validated analytical methods are needed for authentication and quality control. This study aims to quantitatively determine the major iridoids in different parts of noni (fruit, leaf, root, seed, and flower), and comparatively analyse the iridoids in different noni fruits cultivated and collected worldwide, using a validated HPLC-PDA method.

* Correspondence to: Shixin Deng, Research and Development Department, Tahitian Noni International, 737 East, 1180 South, American Fork, UT 84003, USA. E-mail: shixin_deng@tni.com

Research and Development Department, Tahitian Noni International, 737 East, 1180 South, American Fork, UT 84003, USA

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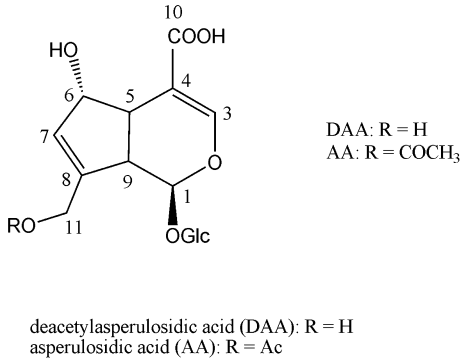

Figure 1. Chemical structures of deacetylasperulosidic acid and asperulosidic acid.

Experimental

Chemicals and Standards

HPLC-grade acetonitrile (MeCN), methanol (MeOH), and water (H_2O) were obtained from Sigma-Aldrich (St Louis, MO, USA). Analytical-grade formic acid was purchased from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ, USA). The chemical standards deacetylasperulosidic acid (DAA) and asperulosidic acid (AA) were isolated from authentic noni fruit in our laboratory. Their identification were determined by HPLC, Mass spectrometry, and NMR (purities > 99%), by comparison with the literature (Kamiya *et al*., 2008). The chemical structures of DAA and AA are listed in Fig. 1. They were accurately weighed and then dissolved in an appropriate volume of MeOH to produce corresponding stock solutions. The working standard solution of DAA and AA for the calibration curve was prepared by diluting the stock solution with MeOH in seven concentration increments from 0.00174 to 1.74 and from 0.0016 to 0.80 mg/mL, respectively. All stock and working solutions were maintained at 0°C in a refrigerator. The calibration curves of the standards were plotted after linear regression of the peak areas vs. concentrations.

Chromatographic conditions and instrumentation

Chromatographic separation was performed on a Waters 2690 separations module coupled with 996 PDA detectors, equipped with a C_{18} column (4.6 × 250 mm; 5 µm, Waters Corporation, Milford, MA, USA). The pump was connected to two mobile phases: (A) MeCN, and (B) 0.1% formic acid in H_2O (v/v), and eluted at a flow rate of 0.8 mL/min. The mobile phase was programmed consecutively in linear gradients as follows: 0–5 min, 0% A (100% B); and 40 min, 30% A (70% B). The PDA detector was monitored in the range of 210–400 nm. The UV λ_{max} of DAA and AA is 235.5 nm, and 235 nm was used for quantitation. The injection volume was 10 µL for each of the sample solutions. The column temperature was maintained at 25°C. Data collection and integration were performed using Waters Millennium software revision 32.

Materials and sample preparation

Fresh noni fruit juice (sample A, Fig. 2) was squeezed from the noni fruit originally collected from the French Polynesia (Tahitian islands). One gram of the fresh fruit juice was diluted with 5 mL of H_2O–MeOH (1:1), and mixed thoroughly; the solution was collected into a 5 mL volumetric flask for HPLC analysis. Dried fruit, seed, root, leaf and flower (samples B-F, Fig. 2) were collected from the Tahitian islands. These were ground into powder, and extracted with MeOH–EtOH (1:1) twice with a sonicator for 30 min each time. The extracts were combined, filtered and then dried in a rotary evaporator under vacuum at 50°C. The dried extracts were re-dissolved with MeOH for HPLC analysis.

The raw noni fruit samples (Fig. 3) were collected from different areas, including the Tahitian islands, Tonga, Dominican Republic, Okinawa, Thailand and Hawaii. The fruit samples were stored below 0°C before use. The fruits were thawed and mashed. Two grams of each mashed fruit was extracted twice with MeOH (125 mL, 30 min each) using a sonicator. The MeOH extract was dried under vacuum in a rotary evaporator. The dried MeOH extracts were re-dissolved with 10 mL of MeOH. Voucher specimens of noni samples are deposited in our laboratory.

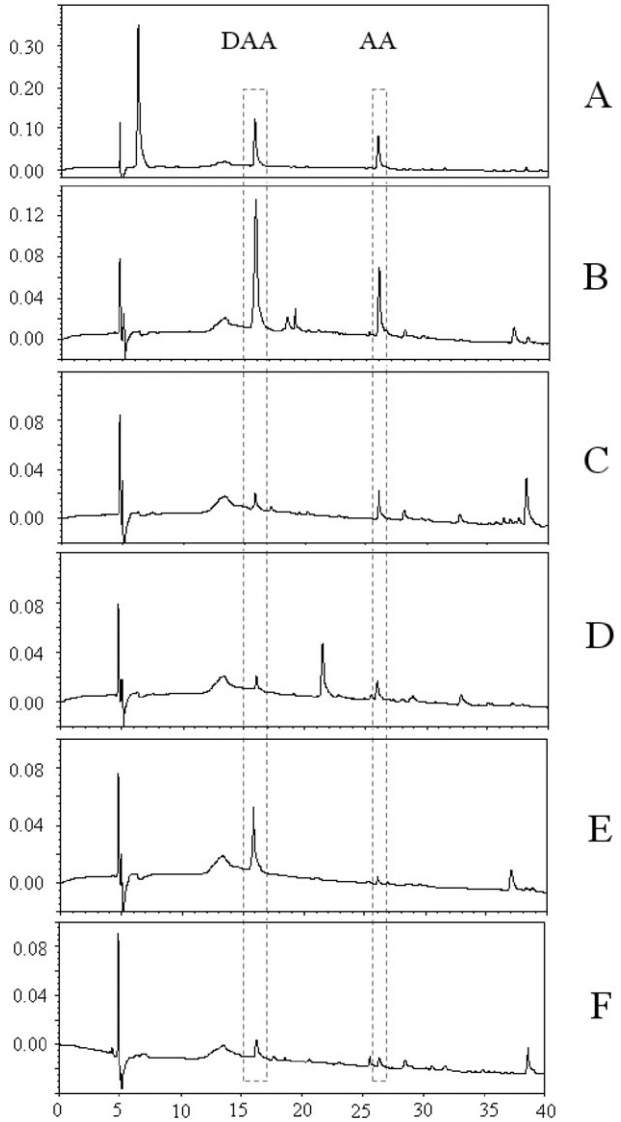

Figure 2. HPLC chromatograms of iridoid analysis in the different parts of noni plants. (A) Noni fruit jiice; (B) dried fruit; (C) leaf; (D) root; (E) seed; (F) flower. DAA, deacetylasperulosidic acid; AA, asperulosidic acid. The *x*- and *y*-axes represent the running time (min) and peak absorbance (AU) respectively.

Analytical method validation

The limits of detection (LOD) and quantitation (LOQ) were defined as the lowest concentrations of analytes in a sample that can be detected and quantified. These LOD and LOQ limits were determined on the basis of signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. The working solutions DAA and AA standards, for LOD and LOQ determinations, were prepared by serial dilution. The intra- and inter-day precision assays, as well as stability tests, were performed by following the method applied to the sample analysis for three consecutive days. Repeatability is the degree of agreement between results, when experimental conditions are maintained as constant as possible, and is expressed as the relative standard deviation (RSD) of replicates.

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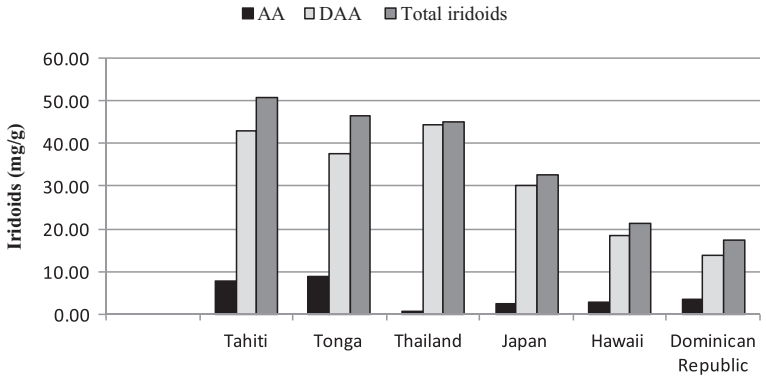

Figure 3. Comparison of iridoid contents in the methanolic extracts of noni fruits collected from different tropical areas worldwide; deacetylasperulosidic acid (DAA); asperulosidic acid (AA); total iridoids (DAA + AA).

Table 1. Chromatographic and spectroscopic characteristics of the iridoids

Compounds	UV λ_{max} (nm)	R_t (min)	LOD (ng)	LOQ (ng)	Linearity range (mg/mL)
DAA[a]	235.5	15.94	10.6	34.8	0.00174–1.74
AA[b]	235.5	26.08	9.7	32.0	0.0016–0.80

[a] Deacetylasperulosidic acid; [b] asperulosidic acid.

Table 2. Intra- and inter-day precisions and stability assays for the quantitative determination of iridoids in noni by HPLC-PDA

Samples	Day 1		Day 2		Day 3		Inter-day	
	Amount detected[a]	RSD (%)	Amount detected[a]	RSD (%)	Amount detected[a]	RSD (%)	Amount detected[a]	RSD (%)
DAA[b]	1.308	0.86	1.291	0.43	1.291	0.62	1.297	0.86
AA[c]	0.276	1.16	0.281	3.00	0.287	1.84	0.281	2.49

[a] Mean ± SD, $n = 3$, mg/mL; [b] deacetylasperulosidic acid; [c] asperulosidic acid.

In the study, intra- and inter-day precisions of the HPLC method were measured by triplicate injections of samples on three consecutive days. Accuracy of the method (recovery) was assessed by the recovery percentage of DAA and AA in the spiked samples. The noni fruit juices were spiked with standards at three different concentrations (equivalent to 50, 100 and 150% concentration of DAA and AA in the samples). The recovery percentage was calculated using the ratio of concentration detected (actual) to those spiked (theoretical). Variation was evaluated by the relative standard deviation (RSD) of triplicate injections in the HPLC experiments.

Results and Discussion

Analytical method validation

The validation of the developed HPLC chromatographic method was conducted on the fresh noni juice to determine LOD, LOQ, linearity, intra-day and inter-day precisions, and accuracy (Tables 1–3). The selected MeCN–H_2O gradient exhibited a good separation and symmetrical peak shapes of target analytes in the HPLC chromatograms. The LODs (S/N = 3) and LOQs (S/N = 10) for DAA and AA were 10.6 and 9.7 ng, and 34.8 and 32.0 ng, respectively. The linear regression equations for DAA and AA were calculated as: $y = 1.443 \times 10^7x - 17342.2$ and $y = 1.537 \times 10^7x - 40804.7$, respectively, where x is the concentration and y is the peak area. The results showed good linearity with correlation coefficients of 0.9994 and 0.9999 for DAA and AA, within the range of concentrations investigated. The intra- and inter-day precisions, as RSDs, of DAA and AA were less than 0.86 and 3.0%, respectively, indicating that DAA and AA were stable during the investigation period. Under the established experimental conditions, percentage recoveries of analytes DAA and AA were from 90.49 to 105.32%, with RSD ranging from 0.40–2.66% (Table 3). The results of the experiments are within tolerance ranges recommended in the guideline for dietary supplement issued by the Association of Analytical Communities (AOAC International, 2002). The characterisation of iridoids DAA and AA in noni samples was conducted by comparing their HPLC retention times and UV maximum absorptions with those of standards (Table 1).

Characterisation and quantitation of DAA and AA in noni different plant parts

Iridoids have been identified in noni fruit, leaf and root previously. In our preliminary experiments, DAA and AA appear to be

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Table 3. Accuracy assays for the quantitative determination of iridoids in noni by HPLC-PDA

Samples	Concentration spiked[a]	Concentration detected[a,b]	Recovery Percentage (%)	RSD %
DAA[c]	0.66	0.619 ± 0.016	93.84	2.66
	1.32	1.271 ± 0.019	96.29	1.53
	2.00	2.106 ± 0.009	105.32	0.40
AA[d]	0.146	0.132 ± 0.002	90.49	1.58
	0.291	0.273 ± 0.004	93.93	1.39
	0.437	0.433 ± 0.004	99.25	0.93

[a] Unit, mg/mL; [b] mean ± SD; $n = 3$; [c] deacetylasperulosidic acid; [d] asperulosidic acid.

Table 4. The concentration of major iridoids in different parts of noni

Samples	DAA[a]	AA[b]
Fruit juice (mg/mL)	1.441 ± 0.027	0.218 ± 0.009
Fruit (dried) (mg/g)	3.741 ± 0.016	1.253 ± 0.005
Leaf (mg/g)	0.338 ± 0.028	0.539 ± 0.007
Root (mg/g)	0.087 ± 0.008	0.326 ± 0.031
Seed (mg/g)	1.303 ± 0.050	0.148 ± 0.011
Flower (mg/g)	0.880 ± 0.040	0.421 ± 0.021

[a] Deacetylasperulosidic acid; [b] asperulosidic acid; mean ± SD; $n = 3$.

the major iridoids in most parts of the noni plant according to their HPLC chromatograms. As such, these two iridoids were employed for the quantitation and comparison of iridoid contents in different noni parts. The typical HPLC chromatograms of noni fruit, leaf, root, seed and flower are shown in Fig. 2. The experimental results (Table 4) indicated that the DAA content in various parts of the plant are, in order of predominance, dried noni fruit > fruit juice > seed > flower > leaf > roots. For AA contents, the rank is dried noni fruit > leaf > flower > root > fruit juice > seed. Among the different plant parts, noni fruit (juice) seems a good source of iridoids. Furthermore, our previous experiments analysed the contents of flavonoids, lignins, anthraquinone and coumarins in noni fruit (Deng *et al*., 2007, 2009, 2010). These studies have demonstrated that the contents of iridoids, specifically deacetylasperulosidic acid and asperulosidic acid, are higher than flavonoids, lignans, anthraquinones and coumarins. Toxicity tests suggested DAA and AA are non-genotoxic in mammalian cells (Nakamura *et al*., 1997). DAA and AA have exhibited many biological activities, including anticlastogenic, antiarthritic, antinociceptive, anti-inflammatory, cardiovascular, cancer-preventive and anti-tumor effects (Nakamura *et al*., 1997; Li *et al*., 2006; Kim *et al*., 2005; Liu *et al*., 2001; Choi *et al*., 2005). As such, these iridoids may contribute to noni's diverse health effects.

Comparison of iridoid contents in noni fruits from different tropical areas

To evaluate the impact of geographical environments (soil, sunlight, temperature, precipitation, etc.) on the iridoid contents in noni fruit, analyses were performed on noni fruits cultivated and collected from different tropical regions worldwide. Ripe noni fruit samples were kept frozen during shipment. Further, MeOH extracts were analysed to control for moisture variations. Figure 3 shows a comparison of DAA, AA and total iridoids (DAA + AA) in different noni fruits. The concentration ranges of DAA and AA in the MeOH extracts were 13.8–42.9 and 0.7–8.9 mg/g, respectively. Moreover, noni fruit collected from French Polynesia had the highest amount of total iridoids, and noni fruit from the Dominican Republic contained the least. The results showed that geographical factors have significant effects on the iridoid contents in noni fruits. As such, different pharmacological activities may be expected for noni fruits collected from different areas.

The impact of pasteurisation on DAA content

Noni fruit juice is usually subjected to heat pasteurisation during commercial processing. Pasteurisation is usually employed in the noni industry, i.e. heating up to 87.7°C for several seconds. In this study, the stability of DAA was conducted. DAA was exposed to 90°C at pH 3.3 for 1 min to determine its thermal stability at acidic conditions. The results indicated that there was no difference in the DAA contents before and after heating, indicating that DAA is stable under the pasteurisation conditions.

Conclusions

A selective analytical HPLC method has been developed and validated for analysis of iridoids in different plant parts and cultivation sources of noni. Iridoids, specifically deacetylasperulosidic acid and asperulosidic acid, are identified as the major components in noni fruit, and also present in leaf, root, seed and flower of the noni plant. Geographical factors seem to influence iridoid content of the fruit. Noni iridoids are stable during pasteurisation. Therefore, the method reported herein may provide an accurate and rapid tool in the qualitative and quantitative analysis of noni and its commercial products.

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