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Discrimination of Three *Pegaga* (*Centella*) Varieties and Determination of Growth-Lighting Effects on Metabolites Content Based on the Chemometry of ^{1}H Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: The metabolites of three species of Apiaceae, also known as *Pegaga*, were analyzed utilizing ^{1}H NMR spectroscopy and multivariate data analysis. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) resolved the species, *Centella asiatica*, *Hydrocotyle bonariensis*, and *Hydrocotyle sibthorpioides*, into three clusters. The saponins, asiaticoside and madecassoside, along with chlorogenic acids were the metabolites that contributed most to the separation. Furthermore, the effects of growth-lighting condition to metabolite contents were also investigated. The extracts of *C. asiatica* grown in full-day light exposure exhibited a stronger radical scavenging activity and contained more triterpenes (asiaticoside and madecassoside), flavonoids, and chlorogenic acids as compared to plants grown in 50% shade. This study established the potential of using a combination of ^{1}H NMR spectroscopy and multivariate data analyses in differentiating three closely related species and the effects of growth lighting, based on their metabolite contents and identification of the markers contributing to their differences.

KEYWORDS: *Pegaga* varieties, growth-lighting effects, metabolites content, chemometry, ^{1}H nuclear magnetic resonance

INTRODUCTION

Pegaga is a popular vegetable in Malaysia, which comprises several varieties of *Centella* and *Hydrocotyle* species from the family Apiaceae. The evergreen creeper is widespread and cultivated in many regions of South East Asia, extending into several subtropical regions. The plant has been reported to grow well in moist and shady places.[1] The common varieties include *Pegaga kampung* (*Centella asiatica*), *Pegaga cina* (*Centella* sp.), and *Pegaga embun* (*Hydrocotyle* sp.). Although *C. asiatica* seems to be related to *Hydrocotyle* species and has been classified in the same subfamily as *Hydrocotyle bonariensis* and *Hydrocotyle sibthorpioides* Lam., its morphological, anatomical, palynological, and phytochemical characteristics retained it in the genus *Centella*.[2] Meanwhile, two species of *Hydrocotyle*, *H. bonariensis* (bigger leaf of 5−10 cm diameter) and *H. sibthorpioides* (smaller leaves of 1 cm diameter), are collectively known as *P. embun* (Figure 1).

Pegaga is often consumed fresh, as a salad and as a healthy drink. It also appears in many herbal preparations for the remedy of a wide range of symptoms such as dysentery, hysteroepilepsy, leprosy, liver complaints, rheumatism, hemorrhoids, and dizziness.[3] Several studies related to the secondary metabolites of *Pegaga* have been reported. Triterpenoidal saponins have been reported from *Bupleurum*, *Centella*, *Hydrocotyle*, and *Sanicula*.[4] Among the *Pegaga* plants, *C. asiatica* has been the most extensively investigated for its biological activities and phytochemical constituents. A number of papers have been published reporting the characteristic constituents such as the triterpenes, asiatic and madecasic acids, and the saponins, asiaticoside and madecassoside. High-performance liquid chromatography (HPLC)[5−7] and liquid chromatography−mass spectrometry (LC-MS)[8] have been the commonly employed analytical methods. Similar oleanane type triterpenoid saponins such as hydrocotylosides I−VII and, more recently, hydrocosisaponins A−F have also been reported in *H. sibthorpioides*.[9,10]

Hydrocotyle and *Centella* species also contain major mono- and sesquiterpenes, such as β-caryophyllene, *trans*-β-farnesene, and germacrene D.[11] Apart from the triterpenes, phenolic constituents such as chlorogenic acids and the flavonoids, kaempferol and quercetin, as well as their glycoside derivatives are commonly found in both *Hydrocotyle* and *Centella* species.[12−14] The evidence of polyacetylenes in the genus *Centella* has also been reported.[15,16] Currently, there is no systematic metabolic characterization of the two genera. Their classification has mainly been via intricate taxonomic classification. Thus, in view of its medicinal value and its commercial popularity as an herbal supplement, differentiating chemical traits between the two genera need to be established.

Metabolomics refer to the comprehensive evaluation of all metabolites, qualitatively and quantitatively, in living organisms. The approach utilizes either an untargeted (NMR or IR) or targeted (MS) analytical tool in combination with multivariate

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analysis to study the metabolome of plant extracts and other biological organisms.[17] Principal component analysis (PCA) has been recognized as a very powerful statistical tool and has been utilized to classify samples according to their total chemical composition.[18−20] Other multivariate analysis such as hierarchical cluster analysis (HCA) has also been used to observe the relationship between the species in the same genus.[21] Metabolomics, specifically ^{1}H NMR-based metabolic fingerprinting, has been applied to a diverse field of plant research, of which classification and quality assessment are among the major interests.[22] Moreover, ^{1}H NMR-based metabolomic approaches have been used in a number of areas such as in environmental chemistry,[23] biotechnology,[24−26] clinical,[27,28] toxicology,[29,30] and agricultural and food chemistry.[31−33]

The aim of the present study was to investigate the potential use of a ^{1}H NMR-based metabolomics approach to discriminate between three varieties of *Pegaga*. In addition, the effects of light on the metabolite contents of *C. asiatica* were explored.

MATERIALS AND METHODS

Chemicals. Analytical-grade methanol and deuterated methanol-d_4 were purchased from Merck (Darmstadt, Germany). Tetramethylsilane (TMS) was purchased from Sigma (St. Louis, MO).

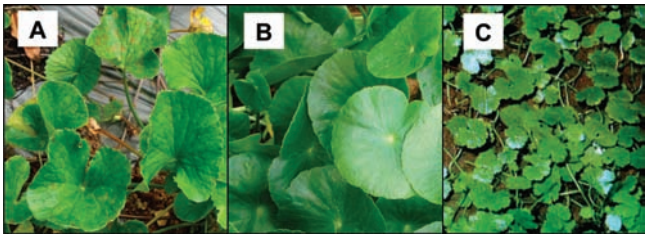

Figure 1. Three varieties of *Pegaga* plants: *C. asiatica* (A), *H. bonariensis* (B), and *H. sibthorpioides* (C).

Plant Materials. Three varieties of *Pegaga* [*C. asiatica* hybrid (A), *H. bonariensis* (B), and *H. sibthorpioides* (C)] were cultivated in the nursery of the Institute of Bioscience, Universiti Putra Malaysia. Large clay pots (100 cm diameter) were filled with growth medium containing a mixture of top soil, organics, and sands (3:2:1 ratio) and 1% cocoa peat. Each large pot constituted an experimental unit. All pots were grown in close proximity and watered during dry spells. Six of these pots were allocated to variety A, 12 to B, and eight to C. The pots were placed under a mesh, which served to block 50% of sunlight, creating a more favorable farm type condition for *Pegaga* development. In addition, for comparison, four pots of *C. asiatica* hybrid were placed under direct light (0% shade). Leaf samples were harvested from each potted plant 4 months after planting, in the middle of January, 2008, for subsequent laboratory procedures. Plant identification was done by a botanist, and voucher specimens of the source material of the three species were deposited in the Herbarium of the Institute of Bioscience, Universiti Putra Malaysia. Measurement of antioxidant activity was carried out using radical scavenger 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to protocols described by Abas et al.[34]

Sample Preparation and Extraction. Approximately 100 g of fresh leaves was collected from each planting plot and dried in the shade. In total, there were six samples for variety A, 12 samples for variety B, and eight samples for variety C. Three subsamples of exactly 1 g each were then taken from each of these samples. The subsamples were subjected to a standard extraction procedure involving Soxhlet extraction for 8 h (7−8 cycles) with 125 mL of 70% (v/v) methanol. Extracts were filtered, evaporated using vacuum rotary evaporator (40−45 °C), and then lyophilized. All prepared extracts were stored at −4 °C prior to NMR analysis.

^{1}H NMR Measurement. In a 1.5 mL vial, 10 mg of each extract was dissolved in 0.7 mL of CD_3OD containing 0.2% TMS and stoppered. The solution was then ultrasonicated at room temperature for 60 min. The solutions were then left to stand overnight, and the suspension was centrifuged at 13000 rpm for 5 min. The supernatants were directly transferred into NMR tubes and subjected to ^{1}H NMR analysis. ^{1}H NMR spectra were acquired at 30 °C on a Varian Unity INOVA

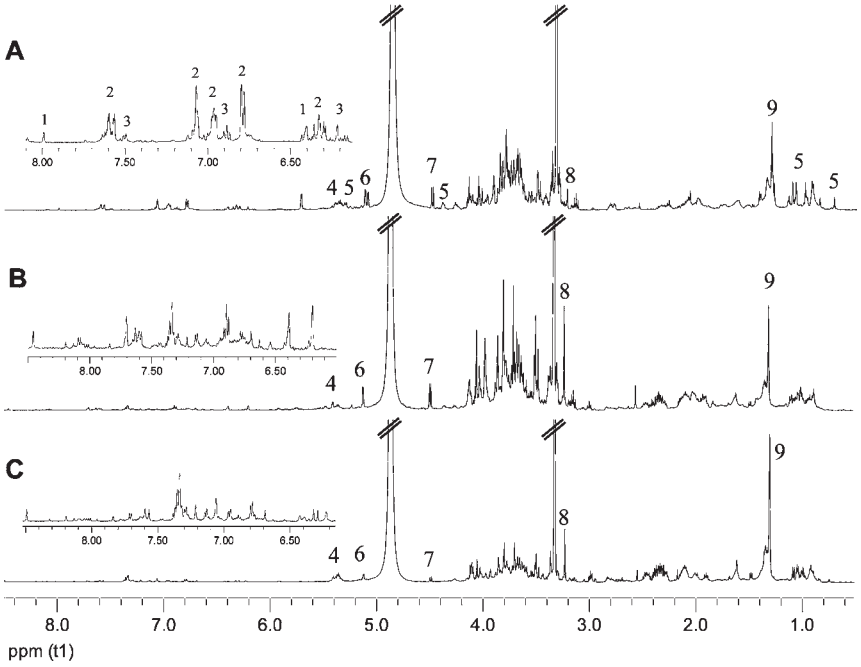

Figure 2. ^{1}H NMR spectra (CD_3OD + 0.2% TMS) of methanolic extract of *H. sibthorpioides* (A), *H. bonariensis* (B), and *C. asiatica* (C). Identified signals: 1, kaempferol derivatives; 2, dicaffeoyl quinic acid derivatives; 3, quercetin derivatives; 4, sucrose; 5, asiaticoside/madecassoside; 6, α-glucose; 7, β-glucose; 8, choline; and 9, fatty acids.

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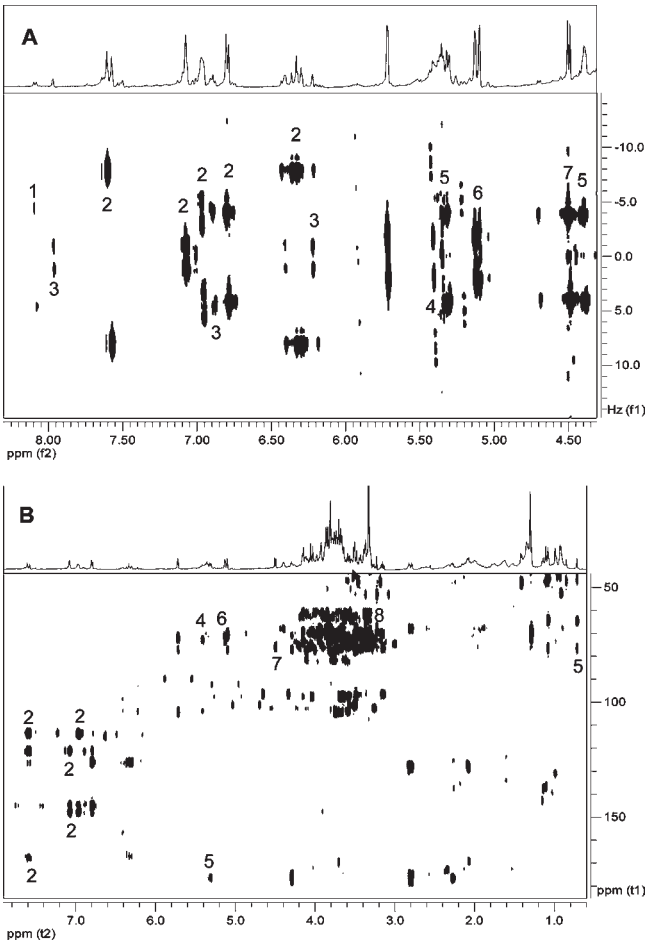

Figure 3. Two-dimensional ^{1}H−^{1}H *J*-resolved (A) and HMBC (B) spectra of *C. asiatica* extract in the region of δ 4.3−9.0. The observed signals are as follows: 1, kaempferol derivatives; 2, dicaffeoyl quinic acid derivatives; 3, quercetin derivatives; 4, sucrose; 5, asiaticoside/madecassoside; 6, α-glucose; 7, β-glucose; and 8, choline.

500 MHz spectrometer (Varian Inc., CA) operating at 499.89 MHz. All spectra were manually phased and baseline corrected. For each sample, 64 scans were recorded using an acquisition time of 193 s, a pulse width of 3.75 μs, and a relaxation delay of 1.0 s. The spectral width was adjusted to a range between −1.00 and 20.00 ppm. TMS was used as an internal standard for chemical shift reference and intensity scaling of all NMR signals.

Data Analysis. The ^{1}H NMR spectra were automatically reduced to ASCII file using Chenomx software (v. 5.1, Alberta, Canada). Spectral intensities were scaled to TMS and binned into regions of 0.04 ppm width for the spectral region δ_H 0.52−10.00, giving a total of 226 integrated regions per NMR spectrum. The signals at δ_H 3.26−3.35 and 4.51−5.03 were excluded from the analysis since these include residual signals from methanol and water, respectively. The averaged binned ^{1}H NMR data were then subjected to PCA and HCA, performed using SIMCA-P+ version 12.0.1.0 (Umetrics AB, Umeå, Sweden). Unit-variance scaling was applied in all analyses. In addition, HCA was carried out using Ward's minimum distance variance. For some constituents, additional support for their identification was obtained using two-dimensional ^{1}H−^{1}H *J*-resolved and heteronuclear multiple-bond correlation (HMBC) experiments.[35]

Relative Quantification. Relative quantification of the identified compounds from the three *Pegaga* varieties was based on the mean peak area of the ^{1}H NMR signals of interest after binning using Chenomx software (v. 5.1).[36] One-way analysis of variance (ANOVA) was conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL) to test significant differences in metabolite contents among the three *Pegaga* varieties, expressed in the signals of interest. Tukey's honest significant difference (Tukey-HSD) multiple-comparison test was performed to determine pairwise differences.[37]

RESULTS AND DISCUSSION

Visual Inspection of ^{1}H NMR Spectra and Assignment of Compounds. Visual inspection of the ^{1}H NMR spectra of the three different *Pegaga* plants showed the presence of different classes of metabolites. These included phenolics, flavonoids, carbohydrates, triterpenes, and fatty acids. Major differences among the extracts were detected in regions δ_H 3.0−5.0 and δ_H 0.5−2.9 ppm, representing carbohydrates and aliphatic constituents, respectively (Figure 2). The interpretation of ^{1}H NMR spectra for the region δ_H 3.0−5.0 was evidently more complex than other regions, because of the overlapping signals.

Differences were also observed in the aromatic region (δ_H 6.0−8.5 ppm) (Figure 2A), which were dominated by signals attributable to dicaffeoylquinic acids and flavonoids. Characteristic signals for the caffeoyl moiety included signals for two *trans* vinyl protons at δ_H 7.61 (d, J = 15.5 Hz, H-7′) and 6.37 ppm (d, J = 15.5 Hz, H-8′), the aromatic ABX spin system at δ_H 7.07 (d, J = 2.0 Hz, H-2′), 6.96 (dd, J = 8.0, 2.0 Hz, H-6′), and 6.78 (d, J = 8.0 Hz, H-5′) (Figure 2A). This identification has also been confirmed by 2D NMR experiments (*J*-resolved and HMBC). *J*-Resolved spectra exhibited characteristic resonance of H-7′, H-8′ of caffeoyl moiety around δ_H 7.61 (d, J = 15.5 Hz) and 6.37 ppm (d, J = 15.5 Hz), respectively, as shown in Figure 3. In addition, HMBC spectra showed ^3J correlation between proton signal at δ_H 7.61 (d, J = 15.5 Hz, H7′) to the carbonyl at around δ_C 168 (C-9′), while proton signal at δ_H 7.61 (d, J = 15.5 Hz, H7′) also revealed the ^2J and ^3J correlations to the aromatic caffeoyl carbons at around δ_C 114−122 (Figure 3).

Apart from the acids, flavonoids (quercetin and kaempferol) were also present, either in their free or glycosylated forms. Signals from quercetin were characterized by the *meta*-coupled signals at δ_H 6.38 (d, J = 2.0 Hz, H-8) and 6.19 (d, J = 2.0 Hz, H-6) (Figure 2A), and the ABX spin system at δ_H 7.64 (dd, J = 8.5, 2.0 Hz, H-6′), 7.73 (d, J = 2.0 Hz, H-2′), and 6.89 (d, J = 8.5 Hz, H-5′). Meanwhile, kaempferol was identified from its AA′XX′ spin system at δ_H 8.09 (d, J = 8.0 Hz, H-2′, H6′) and 6.91 (d, J = 8.0 Hz, H-3′, H-5′) in addition to the *meta*-coupled H-8 and H-6 protons at δ_H 6.38 (d, J = 2.0 Hz) and 6.17 (d, J = 2.0 Hz), respectively. The elucidation of the flavonoids has also been supported by *J*-resolved experiment. In *J*-resolved spectra (Figure 3), the representative H-2′ and H-6′ proton signals of the kaempferol derivatives at δ_H 8.09 (d, J = 8.0 Hz) were observed at low intensity. Moreover, *J*-resolved showed the signals of quercetin at δ_H 7.73 (d, J = 2.0 Hz, H-2′), as well as proton at δ_H 6.38 (d, J = 2.0 Hz, H-8). However, the HMBC spectra were unable to resolve the connectivity of the protons and carbons of the flavonoids due to their low concentrations in the respective extracts.

Further scrutiny of the ^{1}H NMR spectra suggested that di-*O*-caffeoylquinic acids were found in *C. asiatica* extracts, whereas quercetin and kaempferol derivatives are present in all of the extracts. Identification of these components was aided by LC-MS/MS analysis of the *Pegaga* extracts and comparison with literature data.[13] Satake and co-workers[13] reported the isolation of chlorogenic acid and 3,5-di-*O*-caffeoyl quinic acid along with

Table 1. Key ^{1}H NMR Chemical Shifts and Their Respective Coupling Constants of the Metabolites in *Pegaga* Varieties and *p* Values of Tukey-HSD Pairwise Test

		p value[b]		
compd	chemical shifts (ppm) and coupling constants (Hz)[a]	A vs B[c]	A vs C	B vs C
asiaticoside and madecassoside	5.29−5.30 (d, *J* = 8.0 Hz, H-1′), 5.25−5.26 (t, *J* = 3.5 Hz, H-12) 4.39 (d, *J* = 8.0 Hz) 1.28 (d, 6 Hz)	not determined	not determined	not determined
β-glucose	4.49−4.47 (d, *J* = 8.0 Hz)	0.588	<0.001	<0.001
α-glucose	5.11−5.12 (d, *J* = 3.5 Hz)	<0.001	0.001	0.912
sucrose	5.40 (d, *J* = 4.0 Hz) 4.12 (d, *J* = 8.5 Hz)	<0.001	0.05	0.001
fatty acids	1.32 (m), 1.29 (m), 0.85 (t)	0.227	0.001	0.008
choline	3.22 (s)	<0.001	0.001	0.112
di-*O*-caffeoylquinic acid derivatives	7.61 (1H, d, *J* = 15.5 Hz, H-7′) 7.07 (1H, d, *J* = 2.0 Hz, H-2′) 6.96 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′) 6.78 (1H, d, *J* = 8.0 Hz, H-5′) 6.37 (1H, d, *J* = 16.0 Hz, H-8′)	<0.001	<0.001	0.032
kaempferol derivatives	8.09 (2H, d, *J* = 8.0 Hz, H-2′, H6′) 6.91 (2H, d, *J* = 8.0 Hz, H-3′, H-5′) 6.38 (1H, d, *J* = 2.0 Hz, H-8) 6.17 (1H, d, *J* = 2.0 Hz, H-6)	<0.001	<0.001	0.788
quercetin derivatives	7.73 (1H, d, *J* = 1.5 Hz, H-2′) 7.64 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′) 6.89 (1H, d, *J* = 8.5 Hz, H-5′) 6.38 (1H, d, *J* = 1.5 Hz, H-8) 6.19 (1H, d, *J* = 2 Hz, H-6)	<0.001	0.003	0.007

[a] Letters in parentheses indicate the signal multiplicities: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; and m, multiplet. [b] *p* values were results of Tukey-HSD pairwise multiple-comparison tests using SPSS 16.0. Significant level: $p > 0.050$, not significant; $0.050 \geq p > 0.010$, significant*; $0.010 \geq p > 0.001$, very significant**; and $0.001 \geq p$, highly significant***. [c] Letters indicate the three varieties of *Pegaga*: *C. asiatica* (A), *H. bonariensis* (B), and *H. sibthorpioides* (C).

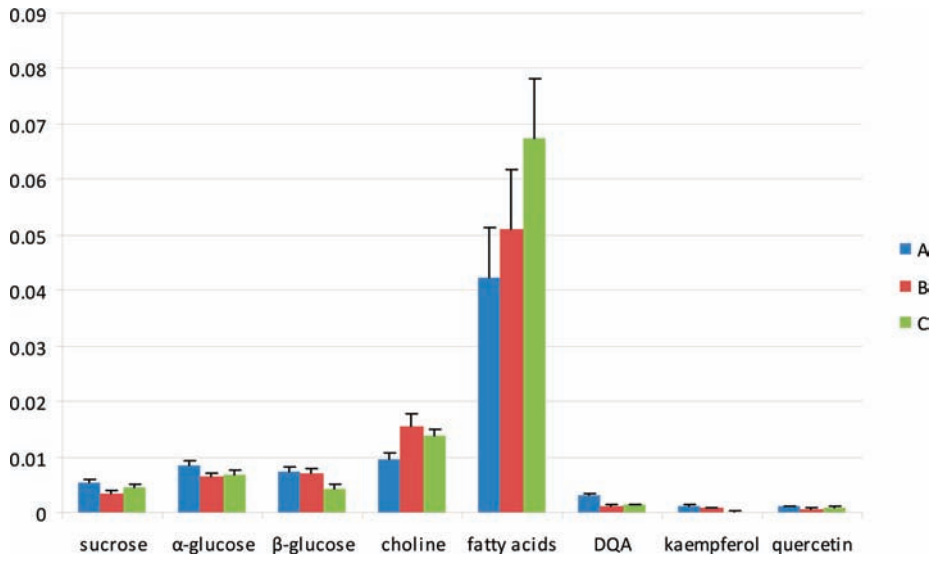

Figure 4. Relative quantification of identified compounds in *Pegaga* extracts [*C. asiatica* (A), *H. bonariensis* (B), and *H. sibthorpioides* (C)] based on the mean peak area of the ^{1}H NMR signals from three extractions associated with the identified compounds. Chemical shifts (δ_H) used for the relative quantification are sucrose at 5.40, *α*-glucose at 5.12, *β*-glucose at 4.48, choline at 3.20, fatty acid at 1.28, dicaffeoyl quinic acid (DQA) at 7.60, kaempferol at 8.08, and quercetin at 7.72. Data presented are the mean (*C. asiatica*, $n = 6$; *H. bonariensis*, $n = 12$; and *H. sibthorpioides*, $n = 8$) ± SD.

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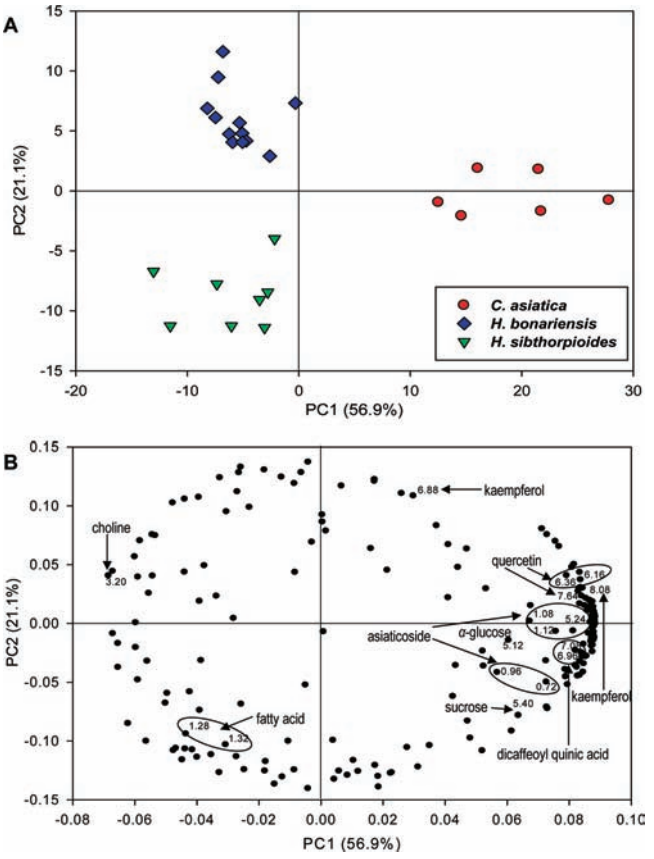

Figure 5. Two-dimensional score (A) and loading (B) plots of the PCA separated by PC1 and PC2 of the methanolic extracts of *C. asiatica*, *H. bonariensis*, and *H. sibthorpioides*. Score plot (A) shows the discrimination of three *Pegaga* varieties. Loading plots (B) indicate the ^{1}H NMR signals of compounds that are responsible for the separation of the three *Pegaga* varieties including sucrose at δ_H 5.40 (d, J = 4.0 Hz); asiaticoside at 5.24 (t, J = 3.5 Hz), 1.12 (s), 1.08 (s), 0.72 (s); dicaffeoyl quinic acid derivatives at 7.08 (d, J = 2.0 Hz), 6.96 (dd, J = 8.0, 2.0 Hz); kaempferol derivatives at 8.08 (d, J = 8.0 Hz), 6.88 (d, J = 8.0 Hz); and quercetin derivatives at 7.64 (dd, J = 8.5, 2.0 Hz), 6.36 (d, J = 1.5 Hz), 6.16 (d, J = 2 Hz).

its isomers, 1,5-di-*O*-caffeoylquinic, 3,4-di-*O*-caffeoylquinic, and 4,5-di-*O*-caffeoylquinic acids. In addition, the flavonoids including kaempferol, quercetin, kaempferol 3-*O*-β-D-glucuronide, and quercetin 3-*O*-β-D-glucuronide were also isolated from the methanolic extract of *C. asiatica*.

The signals for sucrose, α-glucose, β-glucose, choline, and the biomarker triterpenes (asiaticoside and madecassoside) were found in the region of δ_H 3.0−5.4 of ^{1}H NMR spectra (Figure 2A). Identification of the simple sugars was aided by comparison with standard compounds. Among the *Pegaga* species investigated, only the *C. asiatica* contained asiaticoside and madecassoside. A signal at δ_H 5.24 (t, J=3.5 Hz) was assigned to the olefinic proton at H-12, and an anomeric proton signal at δ_H 5.29 (d, J=8.0 Hz, H-1′) was attributed β-glucose of the triterpene. The signal for anomeric proton was shielded due to the presence of acetyl moiety in the former compound. The anomeric proton of the saponins was also detected by the 2D NMR (J-resolved and HMBC), as shown in Figure 3. J-Resolved spectra showed clearly the signal at δ_H 5.29 (d, J = 8.0 Hz), which was assignable to proton H-1′. In the HMBC spectra, the proton signal δ_H 5.29 (d, J = 8.0 Hz) showed correlation to the carbonyl at around δ_C 177 (C-28). The ^{1}H NMR also exhibited the signals for three primary and a secondary hydroxyl groups, typical for the biomarker triterpenes and saponins, which overlapped with the signals for sugar moieties (δ_H 3.0−5.0). These assignments are in accordance with the study of *C. asiatica* leaves, which contained the major triterpenes and their glycosides.[38−40]

The triterpenoid constituents identified from *C. asiatica* are present as the ursane type with double bonds at C12−C13 or C20−C21, and the oleanane type with double bonds occurring at C12−C13 or C13−C18.[40] These triterpenes often contain sugar units at C-28 to form saponins. In addition, oleanane-type triterpenoids having propanoyl substitution at C-21 are also commonly found in *Hydrocotyle* species. The saponins assigned as hydrocotylosides contained three or four sugars at C-28.[9] Other oleanane type saponins such as hydrocosisaponins A−F have also been reported from the *Hydrocotyle* species. The characteristics of these saponins are the attachment of sugar units at C-3 and acetyl substitution at C-21, C-22, and/or C-28.[10]

The region of δ_H 0.5−2.9 was dominated by the characteristic signals for fatty acids [1.29 (m) and 0.85 (t) ppm], as well as aliphatic methylene and methine protons (δ_H 1.46−2.83). The signals for methyls in asiaticoside/madecassoside appeared as four singlets of tertiary methyl groups (δ_H 0.71, 0.85, 1.07, and 1.14), which were assigned to the protons H-24, H-25, H-26, and H-27, respectively. Furthermore, three other signals for secondary methyl groups at δ_H 0.92 and 0.98 which were assigned to H-30 and H-29 of asiaticoside, respectively, and the methyl (δ_H 1.28) of the rhamnoside were also found in this region (Figure 2). All assignments of the ^{1}H NMR signals were accomplished by comparison with ^{1}H NMR spectra of reference compounds and two-dimensional NMR data (J-resolved and HMBC). The ^{1}H NMR assignments and relative quantification of the identified compounds in each *Pegaga* species are shown in Table 1 and Figure 4.

Relative Quantification. To determine the amount of the metabolites identified in the PCA loading plots, relative quantification based on ^{1}H NMR spectral mean peak area was carried out. Variety A was characterized by higher level of sucrose (binned at 5.40 ppm), α-glucose (binned at 5.12 ppm), β-glucose (binned at 4.48 ppm), dicaffeoyl quinic acids (binned at 7.60 ppm), kaempferol derivatives (binned at 8.08 ppm), and quercetin derivatives (binned at 7.72 ppm) as compared to varieties B and C. Fatty acids (binned at 1.28 pm) were found to be more abundant in variety C as compared to varieties A and B. Meanwhile, choline (binned at 3.20 ppm) is higher in variety B in comparison to varieties A and C (Table 1 and Figure 4).

Moreover, according to Tukey-HSD pairwise comparison, carbohydrate contents in variety A, which can be elucidated by the presence of characteristic signals of sucrose, α-glucose, and β-glucose, were significantly different ($p < 0.05$) as compared to varieties B and C. The amount of fatty acids in variety C was also significantly different ($p < 0.05$) as compared to varieties A and B. The presence of choline is significantly higher ($p < 0.05$) in variety B as compared to variety A but not significantly different ($p < 0.05$) when compared with variety C (Table 1).

PCA. The PCA of the methanolic extracts of *C. asiatica* (A), *H. bonariensis* (B), and *H. sibthorpioides* (C) was performed using the entire spectra (δ_H 0.52−10.00). The averaged (from three extractions) ^{1}H NMR data from six samples of *C. asiatica* extracts (A), 12 samples of *H. bonariensis* extracts (B), and eight samples of *H. sibthorpioides* extracts (C) were subjected to PCA analysis. PCA results showed that the first component PC1 contributed 56.9% of the variance, followed by PC2 with 21.1%. Thus, the

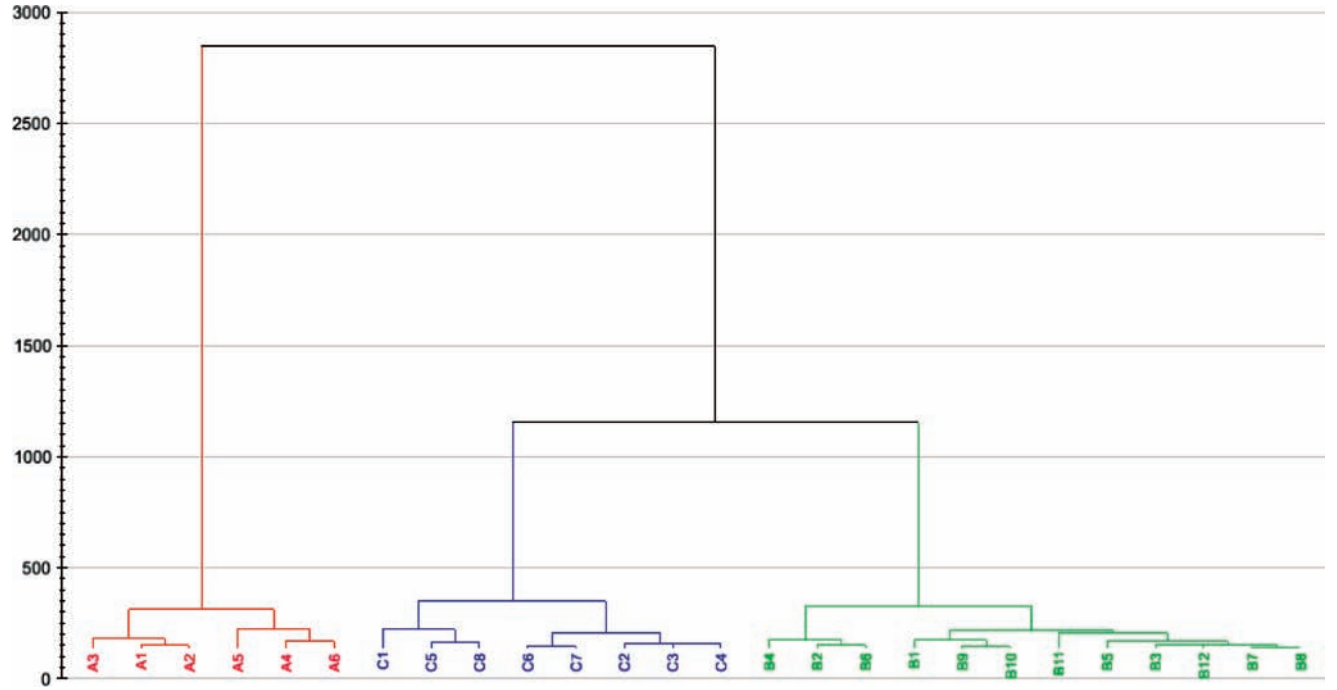

Figure 6. Dendogram of HCA using Ward's minimum variance method of *C. asiatica* (A), *H. bonariensis* (B), and *H. sibthorpioides* (C). In HCA, clustering of samples is based on their similarity.

first two principal components (PCs) cumulatively accounted for 78% of the total variance in the original data. The score plot for PC1 and PC2 was able to separate the *Pegaga* species into three different clusters (Figure 5A).

The loading plot indicated the important chemical shifts contributing to the PCs. In fact, the score and loading plots are complementary to each other. The loadings illustrate the correlations between the new variables, the PCs (PC1, PC2, PC3, etc.), and the original variables (chemical shifts). Furthermore, the position of clusters in a score plot situated in the same dimension in the loading plot is greatly influenced by these variables. The higher the loadings, negative or positive, the more associated the variables are to the PCs. The loading plots of PC1 and PC2 of the three *Pegaga* species are shown in Figure 5B.

According to the loading plot (Figure 5B), fatty acids (binned at δ_H 1.28) were found to be more significant in *H. sibthorpioides* (C) as compared to other extracts. Choline (binned at δ_H 3.20) is shown to be at relatively higher concentration in *H. bonariensis* (B) as compared to other extracts. The loading plot also exhibited several signals for kaempferol (binned at δ_H 8.08 and 6.88), quercetin (binned at δ_H 7.64, 6.36, and 6.16), dicaffeyol quinic acids (binned at δ_H 7.08 and 6.96), and sucrose (binned at δ_H 5.40), which suggested their higher contents in *C. asiatica* (A) as compared to other extracts. Signals for asiaticoside/madecassoside (binned at δ_H 5.24 and δ_H 0.72−1.12) contributed by the olefinic and methyl protons, respectively, were also discernible. Only *C. asiatica* extracts (A) contained the marker compounds saponins, asiaticoside and madecassoside.

HCA. HCA is another unsupervised clustering method to establish the relationships among similar groups of samples. HCA dendrogram of the *Pegaga* extracts was calculated using Ward's minimum variance method, and the dendogram tree was sorted based on size. The HCA results showed that extract B exhibits a closer relationship with C than with extract A. This finding was consistent with the fact that extracts B and C belong to the same genus. The HCA results (Figure 6) were also consistent with the results from the PCA, which separated A in the right region (positive) from B and C in the left region (negative) of PC1 (Figure 5A).

Metabolic Changes in *C. asiatica* Resulting from Different Growth-Lighting Conditions. The PCA score plot (Figure 7A) from the whole ^{1}H NMR spectral data separated *C. asiatica* grown in full daylight from *C. asiatica* grown in 50% shade into two distinguished clusters by PC2. Furthermore, the PCA loading plot revealed that the triterpenes (asiaticoside and madecassoside), as well as the flavonoids (quercetin and kaempferol), and dicaffeoylquinic acids were higher in *C. asiatica* grown in full daylight (Figure 7B). This finding suggests that higher light intensity (0% shade) resulted in an increase in these constituents. The PCA loading plot also showed that fatty acid and choline contributed to the separation, and fatty acid and choline were found to be higher in *C. asiatica* grown in 50% shade. Comparison of the radical scavenging activity (antioxidant) established that extracts from the samples grown in full daylight displayed stronger scavenging activity (IC_{50} of 93.0 ± 2.9 μg/mL) as compared to those grown in 50% shade (IC_{50} of 224.8 ± 6.0 μg/mL). The difference is highly significant ($p < 0.001$) based on statistical ANOVA.

The results suggested that the lighting condition significantly affects the biosynthetic course of a growing plant. In response to light exposure, plants accumulate certain secondary metabolites, such as triterpenoids, flavonoids, and phenolic compounds, which are also well-known to possess antioxidant property.[41] The presence of these compounds, which may act as a sunscreen to protect the cells from injury by UV radiation, may explain the reason for the higher accumulation of these constituents in plants grown under full light exposure as compared to that grown under shade.

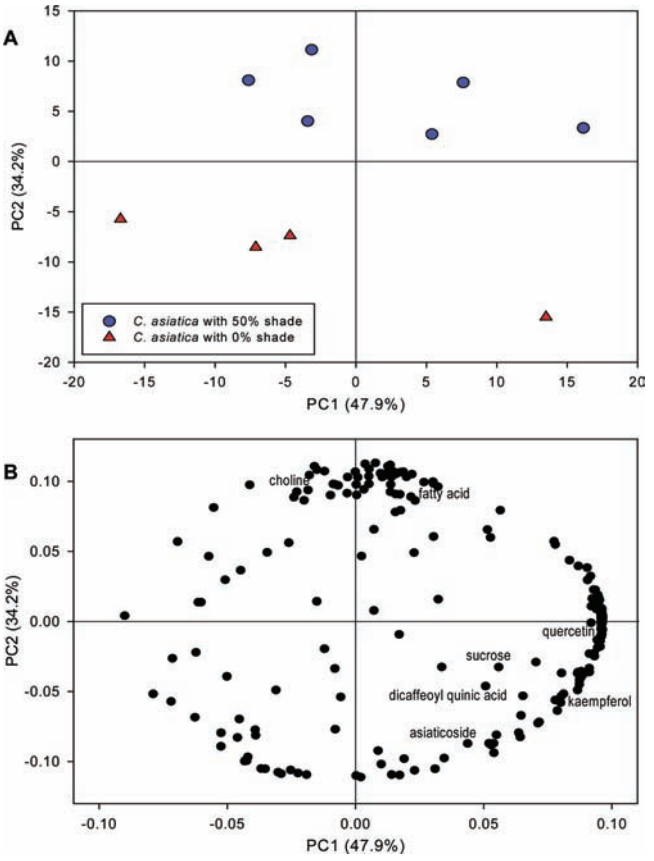

Figure 7. (A) Score and (B) loading plots of the PCA results from the methanolic extracts of *C. asiatica* with 50% daylight and *C. asiatica* with full daylight exposures. The PCA score plot (A) shows clusters between *C. asiatica* grown under 50% shade and that in full daylight, separated by PC2. The loading plot (B) indicates the ^{1}H NMR signals of compounds that contribute to the separation of the two differently grown plants including sucrose at δ_H 5.40 (d, J = 4.0 Hz); asiaticoside at 5.28 (d, J = 8.0 Hz); dicaffeoyl quinic acid derivatives at 7.08 (d, J = 2.0 Hz); kaempferol derivatives at 8.08 (d, J = 8.0 Hz); quercetin derivatives at 7.72 (d, J = 1.5 Hz); choline at 3.20 (s); and fatty acid at 1.28 (m).

C. asiatica has been popularly used traditionally as a tonic to remedy skin diseases and leprosies. Previous investigation has shown that asiatic and ursolic acids significantly suppressed ultraviolet A-induced reactive oxygen species production and lipid peroxidation.[42] The biomarker, asiaticoside, has also been shown to exhibit bioactive properties such as wound healing in in vitro and in vivo assays.[43] In cosmetics, *C. asiatica* extract has been used as an ingredient to regulate visible signs of skin aging.[44]

Metabolite fingerprinting using ^{1}H NMR in combination with multivariate data analysis, PCA and HCA, allowed the discrimination of *Pegaga* varieties into three distinguished clusters. The saponins, asiaticoside and madecassoside, along with chlorogenic acids are the metabolites contributing to the separation of *C. asiatica, H. bonariensis*, and *H. sibthorpioides* extracts. This approach has also allowed discrimination between the varieties based on its metabolite fingerprint. This information will be useful in the selection of the most desirable variety targeted for a specific medicinal use. Elucidation of the effect of growth lighting conditions on metabolites content demonstrated that higher light intensity (0% shade) led to the increase in asiaticoside, madecassoside, flavonoids, and chlorogenic acid contents. This study also suggested that variation in environmental conditions is one of the important factors in causing the variation in chemical metabolites and biological status of an organism.

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