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Chemical characterization and antioxidant activity of three medicinal Apiaceae species

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ABSTRACT

Centella asiatica ('*Pegaga*' = Malaysia) is well known plant that has been used as one of the ingredients in pharmaceutical and cosmetic industries. Using liquid chromatography with diode array and electrospray ionization/mass spectrometry (LC–DAD–ESI/MS) analysis, chemical profiling of three different *Pegaga* extracts (*C. asiatica*, *H. bonariensis* and *H. sibthorpioides*) revealed variations in their metabolite profile. Our findings showed that triterpenes of *C. asiatica* were characterized by the ursane-type triterpenes (madecassoside, asiaticoside, madecassic acid, and asiatic acid), while the two *Hydrocotyle* species consisted of oleanane-type triterpenes (barrigenol derivatives). Other variations are due to the difference in phenolic and flavonoid constituents. The three *Pegaga* extracts were also evaluated for their total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, and xanthine oxidase inhibition (XOI) activities. The results showed that *C. asiatica* has the most potent antioxidant activity (TPC = 72.09 mg/100 g DW; DPPH = 72.99 μg/ml; XOI = 87.68 μg/ml) as compared to *H. bonariensis* (TPC = 28.55 mg/100 g DW; DPPH = 22.43 μg/ml; XOI = 32.23 μg/ml) and *H. sibthorpioides* (TPC = 56.23 mg/100 g DW; DPPH = 37.86 μg/ml; XOI = 14.73. μg/ml).

1. Introduction

Pegaga (Malaysian Pennywort) are perennial creeping plants of the genus *Centella* and *Hydrocotyle* of the Apiaceae family (De Padua and Bunyapraphatsara, 1999). In Malaysia, *Pegaga*, is valued medicinally and also consumed as a traditional vegetable or 'ulam'. Common varieties of *Pegaga* are *Centella asiatica* (asiatic pennywort), *Hydrocotyle bonariensis* (large leaf marsh pennywort), and *Hydrocotyle sibthorpioides* (lawn marsh pennywort). *C. asiatica* has been used since prehistoric time as a herbal remedy for a wide range of applications such as treatment of skin diseases and as a tonic for improving youth, longevity and memory (Burkill, 1966). In the pharmaceuticals industry, *C. asiatica* is a dermatologically effective phytotherapeutic agent that has been used in the form of complex homeopathic preparations, such as an ointment for external application (Brinkhaus et al., 2000). *C. asiatica* has been also used in the cosmetics industry, e.g. as anti-ageing specifically for ameliorating lines and wrinkles, and prophylactically treating loss of elasticity (Oblong and Bissett, 1997), and improving keratinocytes differentiation and for enhancing the epidermal functionality (Sene et al., 2007).

The phytochemistry (Rastogi et al., 1960; Asakawa et al., 1982; Matsuda et al., 2001) and pharmacology (Brinkhaus et al., 2000) of *C. asiatica* has been extensively studied. The main compounds that have been reported to exhibit various physiological effects include its characteristic triterpenoids asiaticoside, madecassoside, asiatic acid and madecassic acid (Gunther and Wagner, 1996). Other classes of compounds such as sterols, polyacetylenes (Govindan et al., 2007), flavonoids and chlorogenic acids (Satake et al., 2007) were also reported to be present in *C. asiatica.*

Despite its popular use, a great deal of uncertainly still exist with regards to the differences between the *Pegaga* varieties, presenting problems in quality control and standardization of downstream products derived from the plants. Earlier, we were able to differentiate between the three *Pegaga* varieties *C. asiatica, H. bonariensis*,

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and *H. sibthorpioides* by using ^{1}H NMR metabolomics approach (Maulidiani et al., 2012). The study revealed that asiaticoside and madecassoside along with chlorogenic acids were the metabolites contributing to the separation of *C. asiatica, H. bonariensis* and *H. sibthorpiodes* extracts. The two triterpene glycosides were only detected in *C. asiatica* extracts but not in *H. bonariensis*, and *H. sibthorpioides*. These compounds are well known to exhibit important biological activities including antioxidant, anticancer and anti-inflammation (Al-Saeedi et al., 2011; Wan et al., 2012).

^{1}H NMR spectroscopy has been used widely as an analytical tool because of its robustness, speed and high-throughput as well as the relatively simple method of sample preparation (Lindon and Nicholson, 1997; Kim et al., 2010). However, MS-based techniques are also widely used as it offers greater speed, sensitivity and selective qualitative and quantitative analyses. Furthermore, efficient identification of each individual constituent is enabled by application of tandem mass (MS/MS) (Schutz et al., 2004; Seeram et al., 2006).

Numerous reports have been published on the quantification of major triterpenoids from *C. asiatica* (asiaticoside, madecassoside, asiatic acid, madecassic acid and their isomers) using HPLC (Gunther and Wagner, 1996; Schaneberg et al., 2003; Rafamantanana et al., 2009). However, there has been only a few reports on the identification of *C. asiatica* constituents using LCMS (Shen et al., 2009). Recently, Long et al. (2012) reported the online identification of two *Centella* species using LCMS. In this paper, we report the on-line identification of three *Pegaga* varieties (*C. asiatica, H. bonariensis*, and *H. sibthorpioides*) using LCMS. The diagnostic fragmentation patterns of chlorogenic acids, flavonoids, and triterpenes in the ESI-MS/MS are discussed on the basis of MS/MS data, in comparison with literature and authentic standards. We further compared the antioxidant and xanthine oxidase inhibitory activities of the three *Pegaga* varieties.

2. Materials and methods

2.1. Chemicals

Ethanol 70% (v/v) used as a solvent for extraction was purchased from ChemPur (Karlsruhe, Germany). HPLC grade acetonitrile (Merck, Darmstadt, Germany) and water purified by a MilliQ system (Millipore, Bedford, MA, USA) were used as mobile phase solvent system in the LCMS analysis. Acetic acid (HOAc) was used as buffer (Fisher, Loughborough, UK). Asiaticoside, madecassoside, asiatic acid, and madecassic acid were purchased from Sigma Chemical Co. (USA), while quercetin and kaempferol were isolated from the dichloromethane fraction of *C. asiatica* and unambiguously identified structurally by ^{1}H NMR analysis and comparison with literature data (Satake et al., 2007). Gallic acid, Folin–Ciocalteu reagent, and sodium carbonate 1,1-diphenyl-2-picrylhydrazyl (DPPH), xanthine oxidase (EC 1.17.3.2), xanthine, potassium phosphate monobasic anhydrous, and allopurinol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Standard compounds 3-*O*-caffeoylquinic acid (chlorogenic acid) and quercetin-3-*O*-rhamnoside were also purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant materials

Leaves of three varieties of *Pegaga* (*C. asiatica*, *H. bonariensis*, and *H. sibthorpioides*) were collected from the plant nursery of the Institute of Bioscience, Universiti Putra Malaysia in October 2008. The three varieties were identified by a Resident Botanist and voucher specimens were deposited at the herbarium of the Laboratory of Natural Products, University of Putra Malaysia.

2.3. Extraction

One gram of the air-dried powdered plant material was soaked in 50 ml of 70% aqueous ethanol (v/v). The suspension was ultrasonicated at room temperature for 60 min, filtered, concentrated in *vacuo* and lyophilized. Extracts were kept at 4 °C prior to analysis.

2.4. HPLC–DAD–ESIMS

Mass spectra were acquired using a Thermo-Finnigan model LCQ^{DECA} (San Jose, CA) ion-trap mass spectrometer equipped with an ESI source interface. Ultrahigh purity helium (He) and high purity nitrogen (N_2) were used as collision and nebulizing gases, respectively. The mass spectra were acquired in both positive and negative ion modes in separate analyses with the following conditions: ESI cone voltage ±4 kV; heated capillary temperature 235 °C, sheath and auxiliary nitrogen gas flows 40 and 18 units. The instrument was coupled to a Surveyor HPLC binary pump, Surveyor diode array detector (DAD) (190–600 nm range; 1 nm bandwidth) and Surveyor autosampler. Chromatographic separation was carried out on an XTerra column (3.5 μm, 150 × 3 mm i.d., Waters Corporation, USA). Acquisition time for the analysis was 80 min using gradient elution with acetonitrile (A) and water containing 0.5% HOAc (B): 0–5 min, 10% A; 5–15 min, 20–25% A; 15–20 min, 25–30% A; 20–35 min, 30% A; 35–40 min, 30–40% A; 40–45 min, 40–55% A; 45–50 min, 55–70% A; 50–55 min, 70–80%A; 55–60 min, 80–90% A; 60–65 min, 90–100% A; 65–80 min, 100%A at a flow-rate of 0.5 ml/min. The concentration of sample extract for each analysis is 5 mg/ml (injection volume: 10 μl). The total ion chromatograms (TIC) were recorded for *m/z* 50 to *m/z* 2000. A data-dependent program was used for further MS/MS analysis. Quantitative determination of antioxidant compounds was carried out *via* HPLC–DAD analysis. Quercetin and quercetin-3-*O*-rhamnoside were selected as the external standards for quantification of flavonoids, whereas 3-*O*-caffeoylquinic acid (chlorogenic acid) for caffeoylquinic acid derivatives. Relative quantification was determined using calibration curves constructed from the standards, prepared in five different concentration levels ranging from 0.1 to 0.006 mg/ml. The calibration curve criteria must be accepted if the $R^2 \geq 0.99$. The relative amount of the compound was expressed as mg/100 g of extract.

2.5. Total phenolic content (TPC)

The total phenolic content (TPC) was determined using Folin-Ciocalteu method (Singleton et al., 1999). In this method, 0.1 ml extract (1 mg/ml) was reacted with 0.1 ml Folin–Ciocalteu reagent and then neutralized with 10 ml of sodium carbonate (7%, v/v; in distilled water). After 60 min of incubation, the absorbance of the solution was measured at 725 nm. Gallic acid was used as a standard and the TPC was expressed as mg of gallic acid equivalents/100 g sample DW.

2.6. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) activity

Radical scavenging activity using >1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was carried out according to protocols described by Mohamad et al. (2004). 100 μl of substock solution (in MeOH) of the extracts at concentrations 250, 125, and 62.5 μg/ml were added into 96-well microplates.5 μl of DPPH reagent (5 mg of DPPH in 2 ml of MeOH) was then added into each well and the solution was kept in the dark for 30 min. The absorbance of the solution was then measured at 517 nm.

Percentage inhibition of DPPH activity was calculated based on the following formula:

$$\frac{(OD_{control} - OD_{samplex}) \times 100\%}{OD_{control}}$$

2.7. Xanthine oxidase inhibitory (XOI) activity

The assay was conducted based on previously reported procedure (Sweeney et al., 2001) with slight modifications. The samples were tested at the concentrations of 200, 100, 50, 25, and 12.5 μg/ml. Percentage inhibition of XOI activity was calculated according to the formula:Percentage inhibition = $(1 - (OD_{sample} - OD_{control})) \times 100\%$

2.8. Statistical analysis

All experiments were repeated thrice and the results reported as mean ± SEM values. Data were analyzed using ANOVA with Tukey-HSD pairwise comparison using Statistical Package for Social Science (SPSS™) software for Windows, Version 16.0 (SPSS Inc., Chicago, IL). A probability level of 5% ($p \leq 0.05$) was considered as significant.

3. Results and discussion

3.1. Liquid chromatography mass spectrometry analysis

The HPLC–DAD chromatograms of the three *Pegaga* variaties (*C. asiatica, H. bonariensis*, and *H. sibthorpioides*) recorded in the UV range of 190–600 nm showed distinctly different metabolite profiles (Fig. 1). The chromatograms of each extract, recorded over 80 min, indicated that the major components were eluted between 5 and 50 min. The TIC profiles showed that most of the prominent peaks detected were attributable to the presence of chlorogenic acids, flavonoids, and triterpene glycosides (Fig. 2). As expected, the triterpenoids, generally having poor chromophores, were poorly detected within the wavelength range of the PDA detector (Fig. 1).

Chlorogenic acids were detected in both *C. asiatica* and *H. sibthorpioides* extracts, whereas flavonoids were present in all of the extracts. Triterpene glycosides were detected in all of the extracts but the aglycones were different for each of the test extract. Table 1 summarizes the retention times (RT), UV, MS and MS/MS data of the detected metabolites.

3.2. Identification of phenolic acids

Chlorogenic acids are a family of esters of hydroxycinnamic acids (caffeic acid, ferulic acid and *p*-coumaric acid) with quinic acids. Based on the negative ion MS/MS data (Table 1), caffeic acid (*m/z* 179) and ferulic acid (*m/z* 193) appear to be the common type of hydroxycinnamic acids for the chlorogenic acids of *C. asiatica* and *H. sibthorpioides*, respectively. Meanwhile, the fragment ion at *m/z* 191 was characteristic of quinic acid. The ions at *m/z* 191 and 179 were considered to be generated from the cleavage of the ester bond linking the two acids. 3-*O*-Caffeoylquinic acid (chlorogenic acid) was observed early at the retention time of 4.91 min (peak **1**) in the TIC profile of both *C. asiatica* and *H. sibthorpioides*. Meanwhile, dicaffeoylquinic acids appear to be major components of the *C. asiatica* extract. This was evident from the negative ion MS/MS data for peaks **11–13**, **15**, and **17** of *C. asiatica* extract (Fig. 2A) which exhibited characteristic ions at *m/z* 191, 353, and 515 (Table 1) attributable to quinic, chlorogenic, and dicaffeoyl quinic acid (cynarin) moieties, respectively. Peaks **11**, **12** and **15** exhibited similar molecular ion peaks at *m/z* 515 indicating that they are possibly isomers, tentatively identified as 1,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid based on comparison with previous reports (Schutz et al., 2004). Peaks **13** and **17** also showed the same molecular ion peak at *m/z* 601 and based on its MS/MS fragmentation with m/z 515, 395 and 353, these were tentatively assigned as isomers of 3,5-di-*O*-caffeoyl-4-malonylquinic acid (Lin and Harnly, 2010).

Possible isomers of dehydrotriferulic acids were identified at the retention times of 35.56 min (peak **29**) and 37.71 min (peak **33**), for *H. sibthorpioides*. The assignments were based on the deprotonated molecular ion peak at *m/z* 577, suggesting a molecular weight of 578 and the fragment ions at *m/z* 383 and 193, corresponding to the loss of one and two ferrulic acid moieties, respectively. The UV maxima at 320 nm shown by both compounds were also characteristic of ferulic acid derivatives and comparison with literature data further supported the assignment (Funk et al., 2005).

3.3. Identification of flavonoid derivatives

Fig. 1 reveals that quercetin and kaempferol were the major flavonols found in the *Pegaga* extracts. Quercetin was characterized by characteristic absorption maxima at 255 and 350 nm, whereas kaempferol was characterized based on its absorption maxima at 260 and 340 nm. In *C. asiatica* extract, three flavonoids could be identified, which were quercetin 3-*O*-β-D-glucuronide, quercetin and kaempferol at RTs = 12.01 min (peak **8**), 27.83 min (peak **24**), and 35.84 min (peak **30**), respectively. Peak **8** exhibited a deprotonated molecular ion at *m/z* 477 with fragment ions at *m/z* 301 for the characteristic loss of the glucuronyl moiety, followed by the typical fragment ions (*m/z* 179 and 151) of free quercetin.

Hydrocotyle species contained flavonoid glycosides as likely chemical markers. Rutin (peak **3**) was identified by the deprotonated molecular ion at *m/z* 609 and the fragment ions at *m/z* 463 (for the loss of rhamnose, $[M\text{-}146]^-$) and 301 (loss of rhamnose and glucose $[M\text{-}146\text{-}162]^-$). Meanwhile peak **4** (RT = 10.25 min) with a deprotonated molecular ion peak at *m/z* 595 was tentatively assigned as quercetin-*O*-pentosyl-hexoside (Singh et al., 2011) based on the fragment ions at *m/z* 463 (loss of pentose moiety [M-132]) and 301 (loss of pentose and glucose $[M\text{-}132\text{-}162]^-$). Peak **6** was assigned as kaempferol-3-*O*-rutinoside (RT = 11.29 min) based on the molecular ion at *m/z* 593 and fragment ions at *m/z* 447 (loss of rhamnose $[M\text{-}H\text{-}146]^-$) and 284 (loss of rhamnose and glucose $[M\text{-}146\text{-}162]^-$. Peak **7** (RT = 11.73 min) was identified as quercetin-3-*O*-glucoside based on the deprotonated molecular ion at *m/z* 463, and fragment ions at *m/z* 445 (loss of H_2O $[M\text{-}18]^-$) and >301 (loss of glucoside). These flavonoid glycosides were also major components of *H. sibthorpioides.* Peak **14** (RT = 15.18 min) present in *H. sibthorpioides* extract exhibited a deprotonated molecular ion at *m/z* 447. Further MS/MS analysis gave a fragment ion at *m/z* 301 for $[M\text{-}H\text{-}146]^-$ arising from a loss of rhamnose yielding the aglycone, quercetin, based on the fragments at m/z 271, 255, 179 and 151. Based on this the compound was assigned as quercetin-3-*O*-rhamnoside.

A larger number of flavonol glycosides were detected in *H. sibthorpioides* extract. A deprotonated molecular ion at *m/z* 741 for peak **2** (RT = 9.08 min) and its fragment ions at *m/z* 609, 447, and 301, which were attributable to loss of pentose $[M\text{-}H\text{-}132]^-$, glucose $[M\text{-}H\text{-}162]^-$ and rhamnose $[M\text{-}H\text{-}146]^-$ from the parent molecular ion, respectively, indicating it to be quercetin-rutinoside with an additional pentose moiety (Ferreres et al., 2010). An isomer of quercetin-rutinoside was also detected as peak **5** (RT = 10.85 min) based on the deprotonated ion peak at *m/z* 609. Peak **10** (RT = 13.92 min) with a deprotonated molecular ion peak at *m/z* at 447, identified as kaempferol-3-*O*-glucoside. Peak **18** (RT = 18.63 min) was identified as quercetin-3-diglucoside based on

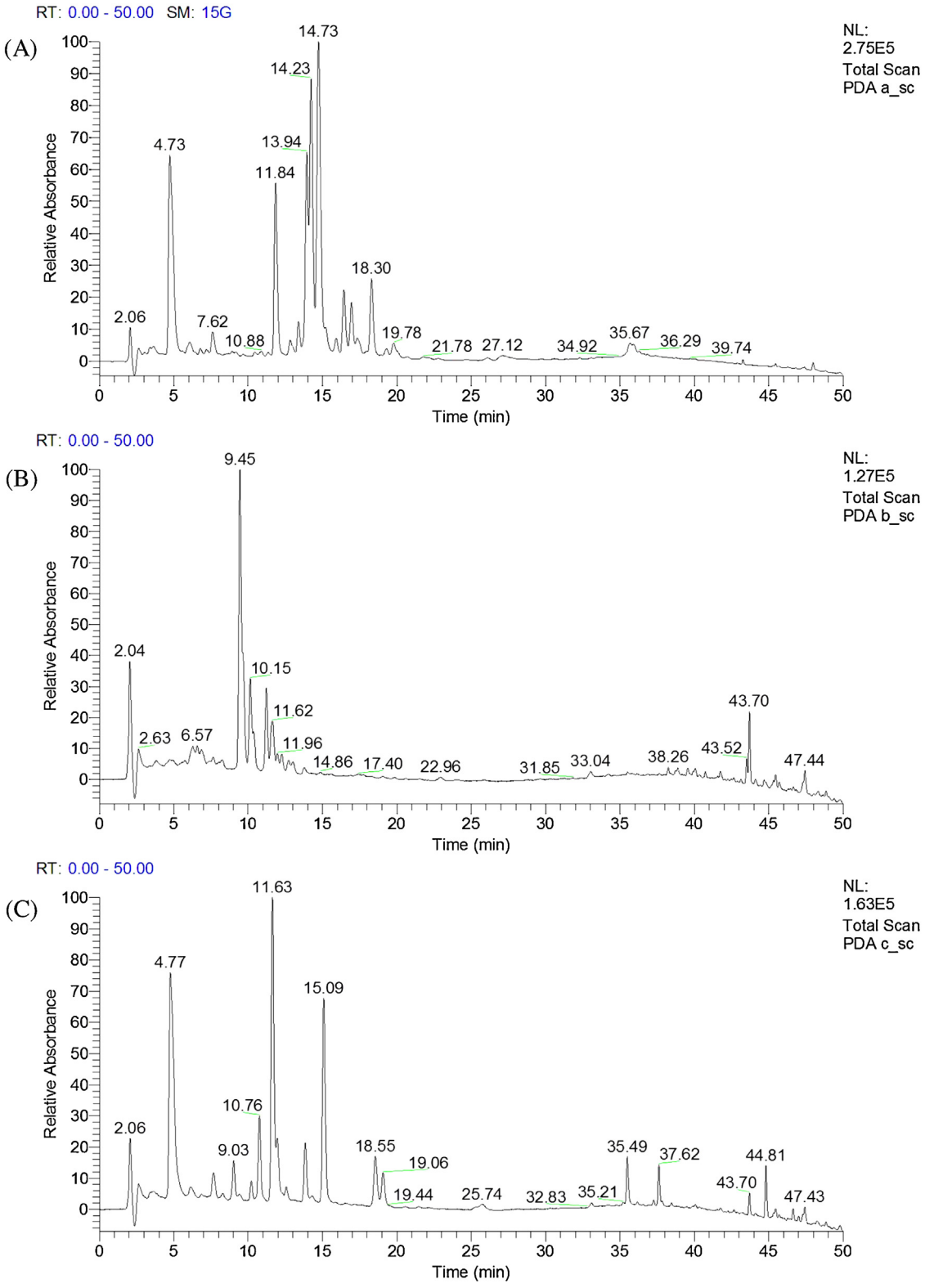


Fig. 1. HPLC–DAD chromatograms of (A) *C. asiatica*, (B) *H. bonariensis*, and (C) *H. sibthorpioides*.

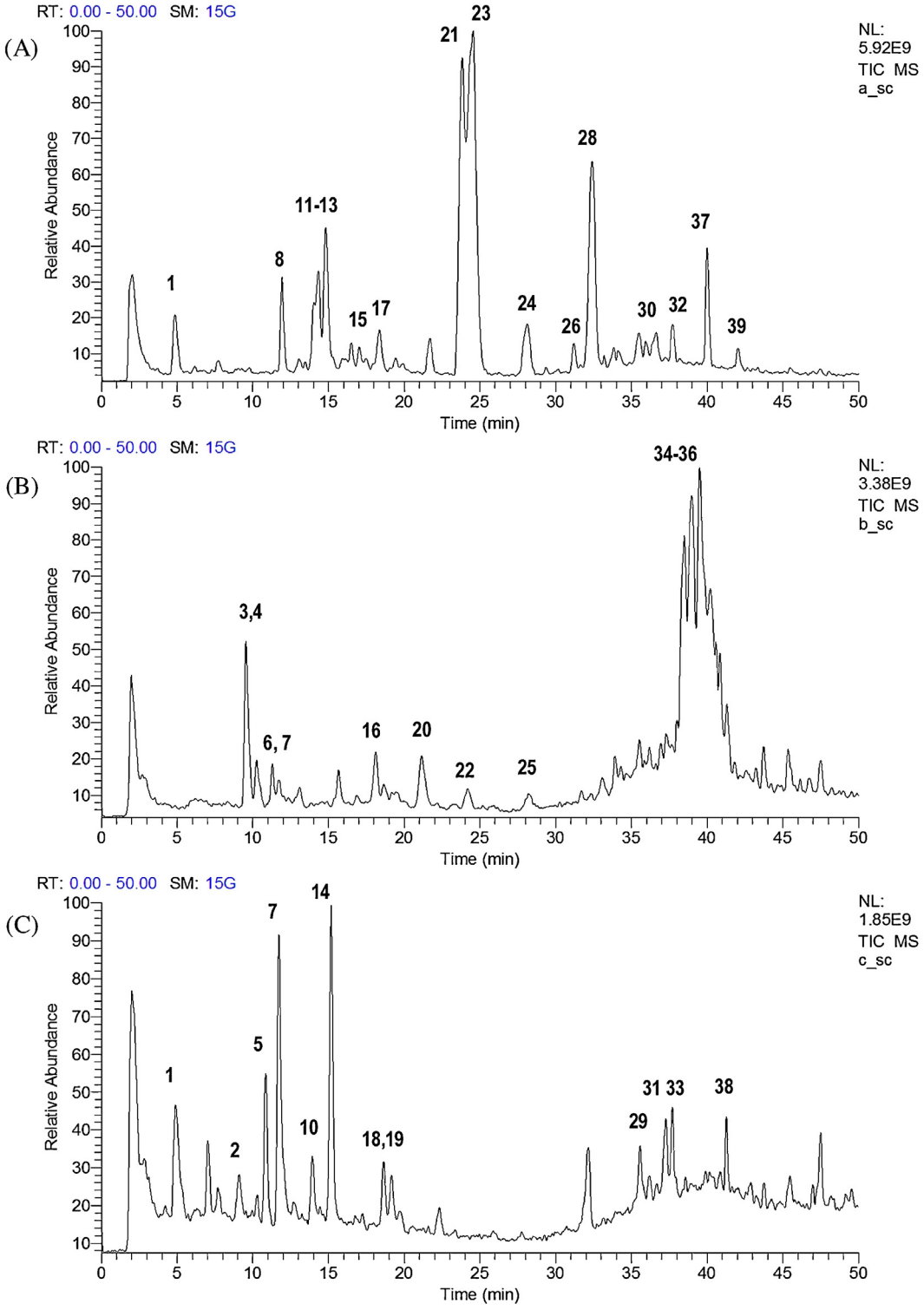


Fig. 2. Total ion chromatograms of (A) *C. asiatica*, (B) *H. bonariensis*, and (C) *H. sibthorpioides*. For peak assignments, see Table 1.

Table 1
Identification of phenolic, flavonol, and triterpenoid constituents in *Pegaga* extracts based on their spectral characteristics obtained from negative ion LC–DAD–ESIMS data.

Peak no.	Retention time (min)	UV (nm)	Parent ion (negative ion)	MS^2 fragments (base ion in bold)	Tentative identity of compound	*Pegaga* extract[a]	Relative amounts of antioxidant compounds[b] (mg/100 g extract)		
							CA	HB	HS
Phenolics									
1	4.91	235, 320	353	**191,** 179, 135	3-*O*-Caffeoylquinic acid (Chlorogenic acid)	CA, HS	0.033 ± 3.284[c]	–	0.018 ± 3.784[c]
11	14.19	245, 325	515	**353**, 335, 191, 179	1,5-Di-*O*-caffeoylquinic acid	CA	0.030 ± 4.323[c]	–	–
12	14.44	245, 325	515	**353**, 335,191, 179	3,5-Di-*O*-caffeoylquinic acid	CA	0.020 ± 4.344[c]	–	–
13	14.99	245, 325	601	557, **515**, 395, 353, 233	3,5-Di-*O*-caffeoyl-4-malonylquinic acid (Irbic acid)	CA	0.023 ± 5.838[c]	–	–
15	16.70	245, 325	515	**353**, 191, 179, 173	4,5-Di-*O*-caffeoylquinic acid	CA	0.007 ± 5.141[c]	–	–
17	18.51	245, 325	601	557, 515, **395**, 245,	3,5-Di-*O*-caffeoyl-4-malonylquinic acid (Irbic acid) isomer	CA	0.006 ± 6.535[c]	–	–
29	35.56	245, 320	577	549, 505, **383,** 355, 313, 193, 163	Dehydrotriferulic acid	HS	–	–	–
33	37.71	250, 320	577	**533**, 505, 383, 355, 341, 298, 193, 163	Dehydrotriferulic acid	HS	–	–	–
Flavonols									
2	9.08	250, 345	741	651, 609, 447, **301**, 271, 255	Quercetin-rutinoside analogue with an additional pentose moiety	HS	–	–	0.048 ± 4.534[d]
3	9.54	255. 350	609	463**, 301**, 271, 255, 179	Quercetin-rutinoside	HB	–	0.423 ± 2.855[d]	–
4	10.25	255, 350	595	463, **301**, 271, 255, 179	Quercetin-*O*- pentosyl-hexoside	HB	–	0.105 ± 3.545[d]	–
5	10.85	255, 350	609	465**, 301**, 271, >255**,** 179	Isomer of quercetin-rutinoside	HS	–	–	0.098 ± 3.831[d]
6	11.29	260, 340	593	447, **284,** 255, 227, 179MS^3 **255**, 163, 151	Kaempferol 3-*O*-glucoside 7-*O*-rhamnoside	HB	–	0.146 ± 2.934[d]	–
7	11.73	255, 350	463	445, **301**, 271, 255, 179	Quercetin-3-O-glucoside	HB, HS	–	0.085 ± 2.572[d]	0.296 ± 1.092[d]
8	12.01	255, 350	477	**301,** 179, 151	Quercetin 3-*O*-b-*D*-glucuronide	CA	0.677 ± 2.804[d]	–	–
10	13.92	260, 340	447	**284**, 255, 227, 179, 151	Kaempferol-3-*O*-glucoside	HS	–	–	0.062 ± 3.290[d]
14	15.18	255, 345	447	**301**, 271, 255, 17**9**, 151	Quercetin-3-*O*-rhamnoside	HS	–	–	0.228 ± 2.273[d]
18	18.63	250, 330	625	**463**, 301, 179	Quercetin-3-diglucoside	HS	–	–	0.040 ± 7.814[d]
19	19.17	260, 335	431	**285**, 255, 227, 191, 179	Kaempferol 3-*O*-rhamnoside	HS	–	–	0.035 ± 8.950[d]
24	27.56	255, 345	301	301, 273, 257, 239, **179**, 151, 125	Quercetin	CA	0.218 ± 1.359[d]	–	–
30	35.84	260, 365	285	**285**, 256, 229, 169,	Kaempferol	CA	0.193 ± 4.313[d]	–	–
Triterpenes									
16	18.12	–	1021.2 [M + HOAc-H] → 961.5	**799.5,** 667.5, 505, 457, 421	Ranuncoside II	HB	–	–	–
20	21.15	–	859 [M + HOAc-H] → 799.5	MS^2 **799.5**, 505 MS^3 **667**, 505, 437, 421	Ranuncoside I	HB	–	–	–
21	23.6	–	1033 [M + HOAc-H] → 973	Full ms **1033**, 973, 929, 769, 736, 624, 504, 469, 392, 347, 291, 256, 179, 119	Madecassoside	CA	–	–	–
22	24.19		946 [M + HOAc-H] → 886	MS^2 813, **751**, 685, >663, 619, 601, 533, 505, 457 MS^3 619, 601, 551, 505	Barrigenol derivatives	HB	–	–	–
23	24.2	–	1033 [M + HOAc-H] → 973	Full ms **1033**, 973, 965, 782, 737, 586, 557, 505, 469, 375, 287,189, 177, 119	Asiaticoside B	CA	–	–	–
25	28.24	–	813 [M + HOAc-H] → 753	MS^2 763, 681, 663, 645, 601, 573, **533**, 505, 455, 421, 389 MS^3 >501, **467**, 456, 422	Barrigenol derivatives	HB	–	–	–

Table 1 (*Continued*)

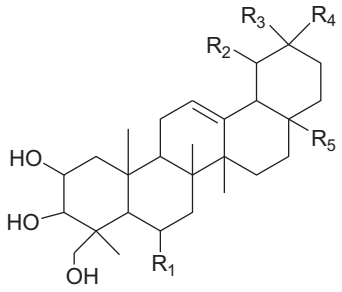
Peak no.	Retention time (min)	UV (nm)	Parent ion (negative ion)	MS^2 fragments (base ion in bold)	Tentative identity of compound	*Pegaga* extract[a]	Relative amounts of antioxidant compounds[b] (mg/100 g extract)		
							CA	HB	HS
26	31.02	–	1017 [M + HOAc-H] → 957	**999**, 903, 791, 678, 565, 452, 338	Centellasaponin A	CA	–	–	–
28	32.26	–	1017 [M + HOAc-H] → 957.5	**999**, 791, 565	Asiaticoside	CA	–	–	–
31	37.27	–	1118	**956**, 893, 793, 775, 757, 731, 685, 660, 569, 551, 456	Hydrocotyloside III	HS	–	–	–
32	37.63	–	1001 [M + HOAc-H] → 941	Full MS **1001**, 979, 941, 885, 798, 601, 547, 477, 283, 179	Asiaticoside D	CA	–	–	–
34	38.48	–	1307 [M + HOAc-H] → 1248	MS^2 **1248**, 1191, 1029, 854, 844 MS^3 **1087**, 729	Ranuncoside IV with addition of glucose unit	HB	–	–	–
35	38.97	–	1145.3 [M + HOAc-H] → 1085	Full MS **1145**, 1085, 996, 923, 853, 787, 731, 643, 588, 529, 506, 495, 483, 357, 275, 179, 173, 132, 119	Ranuncoside IV	HB	–	–	–
36	39.51	–	1145 [M + HOAc-H] → 1085	Full MS **1145**, 1086, 985, 965, 952, 855, 810, 793, 601, 573, 506, 468, 289, 193, 178, 165, 151, 147, 119	Ranuncoside V	HB	–	–	–
37	40.03	–	563 [M + HOAc-H] → 503	MS^2 **505**, 283 MS^3 **435**, 426, 390	Madecassic acid	CA	–	–	–
38	41.27	–	956	MS^2 835, **732**, 714, 617, 569, 549, 524, 456, 319	Hydrocotyloside II	HS	–	–	–
39	41.71	–	547.2 [M + HOAc-H] → 487	MS^2 **487**, 466, 427, 362 MS^3 454, 440, **408**	Asiatic acid	CA			
Unknown									
9	13.09	250, 310	693	**675**, 643, 337, **599**, 547, 369		HB	–	–	–
27	32.16	–	919	799, **720**, 645, 263		HS	–	–	–
40	43.72	240, 285	735 [M + HOAc-H] → 676	675, **659**, 586, 440		HB	–	–	–
41	45.35	260, 340, 405	737 [M + HOAc-H] → 677	719, **674**, 524, 473, 296		HB	–	–	–
42	45.46	255, 425	293	**275**, 236, 171, 163		HS	–	–	–
43	47.51	250, 335	295	295, 277, 185, **171**, 151		HB, HS	–	–	–

[a] *C. asiatica* (CA); *H. bonariensis* (HB); *H. sibthorpioides* (HS).

[b] Value are means ($n = 3$) ± %RSD.

[c] The amount of compound was relative to amount of standard chlorogenic acid, with relationship between peak areas (y) and concentrations (x) was $y = 269319.45x + 1566573.47$ ($R^2 = 0.990$).

[d] The amount of compound was relative to amount of standard quercetin, with relationship between peak areas (y) and concentrations (x) was $y = 237399x - 877718$ ($R^2 = 0.999$).



21 R_1 = OH; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = COO-glc(1-6)glc(1-4)rha

23 R_1 = OH; R_2 = H; R_3 = CH_3; R_4 = CH_3; R_5 = COO-glc(1-6)glc(1-4)rha

26 R_1 = H; R_2 = H; R_3 = CH_3; R_4 = CH_3; R_5 = COO-glc(1-6)glc(1-4)rha

28 R_1 = H; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = COO-glc(1-6)glc(1-4)rha

37 R_1 = OH; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = H

39 R_1 = H; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = H

Fig. 3. Compounds identified from *C. asiatica*.

the deprotonated molecular ion peak at *m/z* 625 and the fragment ions at *m/z* 463 and 301 attributable to two consecutive losses of a glucose moiety. Kaempferol-3-*O*-rhamnoside was also detected in *H. sibthorpioides* extract (peak **19**, RT = 19.17 min) with deprotonated molecular ion at *m/z* 431 and fragment ions at *m/z* 285 characteristic of the aglycone, kaempferol.

3.4. Identification of triterpene glycosides

Pentacyclic triterpenes have been reported to occur in *Centella* species either as *oleanane* or *ursane* types, with the latter being the more common of the two types. Similarly in our LCMS/MS analysis, ursane triterpenes were detected in *C. asiatica* extract. The TIC profile of *C. asiatica* extract showed four major peaks attributable to madecassoside (peak **21**), asiaticoside B (peak **23**), centellasaponin A (peak **26**), asiaticoside (peak **28**), asiaticoside D (peak **32**) and madecassic acid (peak **37**). The structures for these compounds are illustrated in Fig. 3. Identification of the triterpene glycosides in *C. asiatica* extract were supported by comparison with the full MS of authentic standards (madecassoside, asiaticoside, madecassic acid and asiatic acid) as well as by comparison with literature data (Long et al., 2012; Shen et al., 2009). Their full MS spectrum (negative ion mode) showed the pseudo-molecular ions ($[M + HOAc-H]^-$ of each triterpene glycosides, but MS/MS experiments failed to produce any fragment ions. Peak **26** was tentatively identified as the oleanane type triterpene, Centellasaponin A, an isomer of asiaticoside, mainly based on its fragmentation pattern. Additionally, *C. asiatica* exhibited a minor constituent (peak **39**) which exhibited a pseudo-molecular ion peak at *m/z* 547 for $[M + HOAc-H]^-$, assigned as asiatic acid based on its fragmentation pattern and comparison with authentic standard.

In contrast to *C. asiatica*, the triterpene glycosides reported from the *Hydrocotyle* extracts were mostly of the oleanane-type. These findings were in accordance with previous literature (Greca et al., 1994; Matsushita et al., 2004). The TIC profile for *H. bonariensis* extract displayed a pseudo-molecular ion at *m/z* 1021 >$[M + HOAc-H]^-$ for peak **16** (RT = 18.12 min) and MS/MS fragments ions at *m/z* 799 for $[M-H-162]^-$, 667 for $[M-H-162-132]^-$ and 505 for $[M-H-(2 \times 162)-132]^-$, indicating the sequential losses of glucose, arabinose and glucose moieties, respectively. The fragment at *m/z* 505 matched that of the aglycone R_1-barrigenol. Comparison with literature data (Greca et al., 1994) supported the assignment of this component as ranuncoside II.

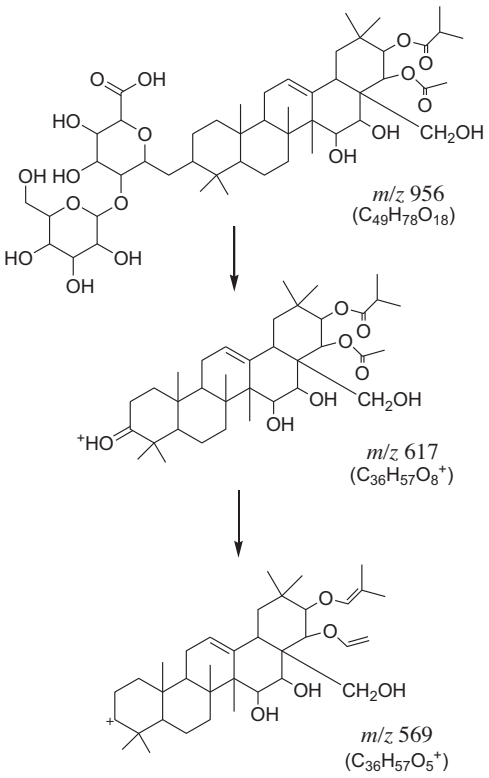


Fig. 4. Proposed mass fragmentation pathway for Hydroctyloside II.

Ranuncoside 1 appeared as peak **20** (RT = 21.15 min) which yielded a pseudo-molecular ion peak at *m/z* 859 ($[M + HOAc-H]^-$) and fragment ion peak at *m/z* 505 in the MS/MS spectra corresponded to its aglycone, resulting from losses of a pentose and a glucose units. The pseudo-molecular ion at *m/z* 1307 ($[M + HOAc-H]^-$) for peak **34** (RT = 38.48 min), indicated a structure consistent with ranuncoside IV (MW 1086) with an additional glucose unit (MW 162). Peak **35** (RT = 38.97 min) and **36** (RT = 39.51 min) were isomeric, showing similar pseudo-molecular ions at *m/z* 1145 $[M + HOAc-H]^-$. They were tentatively assigned as ranuncoside IV and ranuncoside V (Greca et al., 1994), respectively, based on their full MS data. The presence of other ranuncosides was also detected in *H. bonariensis*. Several other triterpene glycosides with R_1-barrigenol as its aglycone were also detected in *H. bonariensis*. These are peaks **22** (RT = 24.19 min) and **25** (RT = 28.24 min), which gave pseudo-molecular ions at *m/z* 946 $[M + HOAc-H]^-$ and 813 $[M + HOAc-H]^-$, respectively.

Oleanane glycosides were also detected in *H. sibthorpioides* extract with barrigenol as its aglycone, but with propanoyl or methylpropanoyl and acetyl groups attached at C21 and C22, respectively (Matsushita et al., 2004). Peak **38** (RT = 41.27 min) gave a deprotonated molecular ion peak at *m/z* 956 and fragment ion at *m/z* 617 corresponding to the losses of a glucose and a hexosyluronic acid $[M-H-162-176]^-$. Furthermore, the MS/MS showed a fragment ion at *m/z* 569 indicating the barrigenol aglycone minus a methoxy group and water $[M-H-OCH_3-H_2O]^-$. Thus, the compound was assigned as hydrocotyloside II (Fig. 4). Peak **31** (RT = 37.27 min) which gave a deprotonated molecular ion at *m/z* 1118 and a fragment ion at *m/z* 956 for $[M-H-162]^-$ was assigned as hydrocotyloside III. The retention times, UV, MS and MS^2 data obtained by HPLC–DAD–ESIMS, as well as the relative amounts of antioxidant compounds of each of the *Pegaga* varieties are presented in Table 1.

Table 2
Total phenolic content (TPC), radical scavenging activity (RSA), and xanthine oxidase inhibitory (XOI) activities of three *Pegaga* extracts.

Sample	TPC (mg GAE/100 g DW)[a,b]	DPPH-RSA (at 250 μg/ml)[a]	XOI (at 200 μg/ml)[a]
C. asiatica	72.09 ± 2.45	72.99 ± 0.42	87.68 ± 4.08
H. bonariensis	28.55 ± 1.14	22.43 ± 0.90	32.23 ± 1.28
H. sibthorpioides	56.23 ± 2.33	37.86 ± 0.49	14.73 ± 1.06
Allopurinol (10 μM)			84.68 ± 1.25
Quercetin (50 μM)		87.63 ± 1.63	

[a] The experiment was conducted in triplicates and the result was calculated as mean ± SEM.
[b] For TPC assay, the curve absorbance of standard gallic acid versus concentrations is described by the equation $y = 0.0059x + 0.0291$ ($R^2 = 0.996$).

3.5. Determination of the total phenolic content and antioxidant activity

Phenolic compounds are often correlated to the antioxidant properties of a plant due to their capability as electron donors in free radical reactions. In this study, using the Folin-Ciocalteu method, it was shown that there are significant differences ($p < 0.05$) in the TPCs of the *Pegaga* extracts. The TPCs of the extracts ranged from 28.6 to 77.9 mg GAE/100 g DW with *C. asiatica* showing the highest value of 77.9 mg GAE/100 g DW, followed by *H. sibthorpioides* and *H. bonariensis* with TPC values of 58.2 and 28.6 mg GAE/100 g DW, respectively (Table 2).

Reactive oxygen species (ROS) play an important role in ageing and age-related diseases. Thus, taking antioxidants as supplements are expected to slow down the ageing process. In this study, determination of the antioxidant activities of the three *Pegaga* extracts (*C. asiatica, H. bonariensis*, and *H. sibthorpioides*) were carried out based on DPPH radical scavenging activity (RSA) and xanthine oxidase inhibitory assays. The mechanism involved in DPPH assay is that the stable free radical (DPPH) will react with antioxidants to form 1,1-diphenyl-2-picrylhydrazine. The presence of free radical-scavenger results in the disappearance of color and therefore its high radical scavenging percentage represents high antioxidant activity. Adopting this experiment to our samples, *C. asiatica* extract was found to be the most potent radical scavenger towards DPPH with IC_{50} value of 180.3 μg/ml, while extracts *H. bonariensis* and *H. sibthorpioides* showed weaker activity with IC_{50} value of >250.0 μg/ml. Further ANOVA test with Tukey-HSD pairwise comparison showed that there is significant difference ($p < 0.05$) in DPPH-RSA of *C. asiatica* when compared to *H. bonariensis* and *H. sibthorpioides* extracts (Table 2). The antioxidant results were in good correlation with the TPC values. These results were also supported by quantitative determination of antioxidant compounds using HPLC–DAD quantitative analyses. *C. asiatica* extract has the highest amounts of phenolics (0.119 mg/100 g of extract in the forms of caffeoylquinic acid derivatives) and flavonoids (1.088 mg/100 g of extract), followed by *H. sibthorpioides* with total phenolics of 0.018 mg/100 g of extract and total flavonoids of 0.807 mg/100 g of extract, and *H. bonariensis* extracts with total flavonoids of 0.759 mg/100 g of extract (Table 1). According to our previous study on the application of Partial Least Squares model for the prediction of antioxidant activity in *Pegaga* extracts from ^{1}H NMR spectroscopy, dicaffeoylquinic acid derivatives were the constituents that gave the most contribution to the DPPH-RSA, followed by quercetin and kaempferol derivatives (Maulidiani et al., 2013).

Xanthine oxidase (XO) is a key enzyme that catalyzes the last two steps of purine catabolism, the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Elevation of uric acid level in the blood will leads to the deposition of urate crystals in joints and kidneys causing hyperuricemia and gout arthritis. The results from the assay showed that *C. asiatica* extract inhibited 87.7% XO activity at the concentration of extract 200 μg/ml, while the two species of *Hydrocotyle* showed weak inhibitory activity (Table 2). Furthermore, ANOVA test with Tukey-HSD pairwise comparison showed that there is significant ($p < 0.05$) difference of XO inhibitory activity between *C. asiatica* and the other two *Hydrocotyle* extracts (Table 2).

4. Conclusion

In conclusion, identification of the three *Pegaga* (*C. asiatica*, *H. bonariensis*, and *H. sibthorpioides*) varieties using LC–DAD–ESIMS/MS technique allowed us to characterize the chemical constituents of the plants based on their MS data and MS/MS fragmentation pattern. In addition to quantification of the total phenolic content, the identity of the chlorogenic acids, flavonoids, and triterpenes composition in the three varieties was also determined. Antioxidant evaluation (DPPH-RSA, and XOI activities) of the extracts showed that the potency correlated well with the total phenolic content (TPC) and revealed that *C. asiatica* extract was the most active compared to *H. bonariensis*, and *H. sibthorpioides*. Based on this observation, with respect to the medicinal use of *Pegaga* as an antioxidant agent, *C. asiatica* would be the more preferred species. In addition, LC–DAD–ESI/MS analysis of the *Pegaga* extracts, might be one of the alternative solution for quality control and standardization of the plants.

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References

Al-Saeedi, F.J., Bitar, M., Pariyani, S., 2011. Effect of asiaticoside on 99mTc-tetrofosmin and 99mTc-sestamibi uptake in MCF-7 cells. J. Nucl. Med. Technol. 39, 279–283.
Asakawa, Y., Matsuda, R., Takemoto, T., 1982. Mono- and sesquiterpenoids from *Hydrocotyle* and *Centella* species. Phytochemistry 21, 2590–2592.
Brinkhaus, B., Lindner, M., Schuppan, D., Hahn, E.G., 2000. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomedicine 7, 427–448.
Burkill, I.H., 1966. Dictionary of the Economic Products of the Malay Peninsula. Ministry of Agriculture and Cooperatives, Kuala Lumpur, Malaysia.
De Padua, L.S., Bunyapraphatsara, N., 1999. Medicinal and Poisonous Plants 1. In Plant Resources of South-East Asia. Backhuys, Leiden, The Netherlands.
Ferreres, F., Taveira, M., Pereira, D.M., Valentao, P., Andrade, P.B., 2010. Tomato (*Lycopersicon esculentum*) seeds: new flavonols and cytotoxic effect. J. Agric. Food Chem. 58, 2854–2861.
Funk, C., Ralph, J., Steinhart, H., Bunzel, M., 2005. Isolation and structural characterisation of 8–O–4/8–O–4- and 8–8/8–O–4-coupled dehydrotriferulic acids from maize bran. Phytochemistry 66, 363–371.
Govindan, G., Sambandan, T.G., Govindan, M., Sinskey, A., Vanessendelft, J., Adenan, I., Rha, C.K., 2007. A bioactive polyacetylene compound isolated from *Centella asiatica*. Planta Med. 73, 597–599.
Greca, M.D., Fiorentino, A., Monaco, P., Previtera, L., 1994. Oleanane glycosides from Hydrocotyle ranunculoides. Phytochemistry 36, 1479–1483.
Gunther, B., Wagner, H., 1996. Quantitative determination of triterpenes in extracts and phytopreparations of *Centella asiatica* (L.) Urban. Phytomedicine 3, 59–65.
Kim, H.K., Choi, Y.H., Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. Nat. Protoc. 5, 536–549.
Lin, L.-Z., Harnly, J.M., 2010. Identification of the phenolic components of chrysanthemum flower (*Chrysanthemum morifolium* Ramat). Food Chem. 120, 319–326.
Lindon, J.C., Nicholson, J.K., 1997. Recent advances in high-resolution NMR spectroscopic methods in bioanalytical chemistry. TrAC, Trends Anal. Chem. 16, 190–200.
Long, H.S., Stander, M.A., Van Wyk, B.E., 2012. Notes on the occurrence and significance of triterpenoids (asiaticoside and related compounds) and caffeoylquinic acids in *Centella* species. S. Afr. J. Bot. 82, 53–59.
Matsuda, H., Morikawa, T., Ueda, H., Yoshikawa, M., 2001. Medicinal foodstuffs. XXVII. Saponin constituents of gotu kola (2): structures of new ursane- and oleanane-type triterpene oligoglycosides, centellasaponins B, C, and D, from *Centella asiatica* cultivated in Sri Lanka. Chem. Pharm. Bull. 49, 1368–1371.

Matsushita, A., Sasaki, Y., Warashina, T., Miyase, T., Noguchi, H., Velde, D.V., 2004. Hydrocotylosides I–VII, New oleanane saponins from *Hydrocotyle sibthorpioides*. J. Nat. Prod. 67, 384–388.

Maulidiani, H., Khatib, A., Shaari, K., Abas, F., Shitan, M., Kneer, R., Neto, V., Lajis, N.H., 2012. 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. J. Agric. Food Chem. 60, 410–417.

Maulidiani, Abas, F., Khatib, A., Shitan, M., Shaari, K., Lajis, N.H., 2013. Comparison of partial least squares and artificial neural network for the prediction of antioxidant activity in extract of *Pegaga* (*Centella*) varieties from ^{1}H nuclear magnetic resonance spectroscopy. Food Res. Int. 54, 852–860.

Mohamad, H., Abas, F., Permana, D., Lajis, N.H., Ali, A.M., Sukari, M.A., Hin, T.Y.Y., Kikuzaki, H., Nakatani, N., 2004. DPPH free radical scavenger components from the fruits of *Alpinia rafflesiana* Wall. ex Bak. (Zingiberaceae). Z. Naturforsch. C J. Biosci. 59, 811–815.

Oblong, J.E., Bissett, D.L., 1997. Methods of regulating skin condition with Centella asiatica extract. In: Patent Cooperation Treaty. W. I. P. Organization, pp. 1–45.

Rafamantanana, M.H., Rozet, E., Raoelison, G.E., Cheuk, K., Ratsimamanga, S.U., Hubert, P., Quetin-Leclercq, J., 2009. An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (APIACEAE). J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877, 2396–2402.

Rastogi, R.P., Sakar, B., Dhar, M.L., 1960. Chemical examination of *Centella asiatica* Linn Part I. Isolation of the chemical constituents. J. Sci. Ind. Res. 19, 252–257.

Satake, T., Kamiya, K., An, Y., Oishi Nee Taka, T., Yamamoto, J., 2007. The antithrombotic active constituents from *Centella asiatica*. Biol. Pharm. Bull. 30, 935–940.

Schaneberg, B.T., Mikell, J.R., Bedir, E., Khan, I.A., 2003. An improved HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products. Pharmazie 58, 381–384.

Schutz, K., Kammerer, D., Carle, R., Schieber, A., 2004. Identification and quantification of caffeoylquinic acids and flavonoids from artichoke (*Cynara scolymus* L.) heads, juice, and pomace by HPLC-DAD-ESI/MSn. J. Agric. Food Chem. 52, 4090–4096.

Seeram, N.P., Lee, R., Scheuller, H.S., Heber, D., 2006. Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. Food Chem. 97, 1–11.

Sene, G., Loiseau, A., Lepetit, J., 2007. Use of compounds from Centella asiatica. In: Patent Cooperation Treaty. W. P. I. Organization, pp. 1–18.

Shen, Y., Liu, A., Ye, M., Wang, L., Chen, J., Wang, X.R., Han, C., 2009. Analysis of biologically active constituents in *Centella asiatica* by microwave-assisted extraction combined with LC-MS. Chromatographia 70, 431–438.

Singh, A.P., Wilson, T., Luthria, D., Freeman, M.R., Scott, R.M., Bilenker, D., Shah, S., Somasundaram, S., Vorsa, N., 2011. LC–MS–MS characterisation of curry leaf flavonols and antioxidant activity. Food Chem. 127, 80–85.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., Lester, P., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 299, 152–178.

Sweeney, A.P., Wyllie, S.G., Shalliker, R.A., Markham, J.L., 2001. Xanthine oxidase inhibitory activity of selected Australian native plants. J. Ethnopharmacol. 75, 273–277.

Wan, J., Gong, X., Jiang, R., Zhang, Z., Zhang, L., 2012. Antipyretic and anti-inflammatory effects of asiaticoside in lipopolysaccharide-treated rat through up-regulation of heme oxygenase-1. Phytother. Res. 27, 1136–1142.