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Polyphenols and antioxidant activities of selected traditional vegetables

(Polifenol dan aktiviti antioksida ulam-ulam terpilih)

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Keywords: polyphenols, *ulam*, antioxidant activity

Abstract

Consumption of fruits and vegetables is associated with a lower risk of serious diseases such as cardiovascular disease and cancer. Fruits and vegetables are also widely reported as good sources of dietary antioxidants. Five selected Malaysian traditional vegetables, namely, *Centella asiatica* (L.) Urban (*pegaga*), *Anacardium occidentale* L. (*gajus*), *Colubrina asiatica* (*peria pantai*), *Pluchea indica* (*beluntas*) and *Premna cordifolia* (*bebuas*) from MARDI's gene bank, Seberang Perai were screened for phenolic compounds and analysed for antioxidant activities using a Ferric Reducing Antioxidant Potential (FRAP) assay in two batches of sampling. Total phenolic content varied from 100–415 mg/kg gallic acid equivalent (GAE) in batch 1 and from 62–386 mg/kg GAE in batch 2. *Anacardium occidentale* had the highest total phenolic content followed by *P. indica* B and A, *P. cordifolia, C. asiatica* and the lowest was *C. asiatica*. Total antioxidant activity indicated that *A. occidentale* showed the highest activity which was probably due to these phenolics. Flavonol glycosides were predominant in most of the species, particularly *A. occidentale* with levels ranging 6.4–12.4 ± 0.3 mg/g fresh weight (fw). Chlorogenic acids were the main components identified and quantified in *C. asiatica* and *P. indica*. In this study, it was shown that the total phenolic content of plant extracts was positively correlated with total antioxidant capacity.

Introduction

In Malaysia, traditional vegetables or 'ulam' are very nutritious when consumed fresh or cooked at a medium temperature as they contain a lot of vitamins, fibres and minerals. They have been reported to have medicinal properties against diabetes, heart disease and problems with the digestive tract (Mustafa 1994). The nutritional components of the species used in the present study have been reported by Saidin (2000).

Nutritional elements in the diet play a major role in contributing to human health. Consumption of fruits and vegetables is good for health and could lower the probability of getting serious diseases. Fruits and vegetables are rich sources of dietary antioxidant and nutrients which play an

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important role in protecting humans against chronic diseases such as cardiovascular disease (CVD) and cancers (WHO 2003). They also have a variety of biological properties which have been linked to disease prevention (Hollman et al. 1996).

Fruits and vegetables are documented to be high in antioxidants which delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidising chain reactions by free radicals, and therefore may reduce oxidative damage to the human body (Hollman and Arts 2000; Ismail et al. 2004). The occurrence of such oxidative damage is believed to be a significant causative factor in the development of chronic diseases such as cancer and CVD (Proteggente et al. 2003; Arts and Hollman 2005). Therefore, the risk of these diseases could be reduced by increasing daily intake of fruits and vegetables such as broccoli, spinach, shallots, potatoes and carrots which are rich sources of antioxidants (Cao et al. 1996; Heim et al. 2002).

Apart from the antioxidant vitamins, fruits and vegetables also contain carotenoids, polyphenols and non-antioxidant vitamins, which are also responsible for the protective effects against cancer and CVD (Heim et al. 2002; Tucker 2003).

One of the major compounds in fruits and vegetables is phenol. Polyphenolic compounds are secondary metabolites widespread in the plant kingdom. They form an integral part of the human diet with fruits, vegetables, tea and red wine providing an especially abundant source. During the past decade, interest has arisen in these compounds as there is some evidence to suggest that an increased consumption of phenolic rich food and beverages may help to prevent diseases. Polyphenols are reducing agents and their potential health-related properties have been ascribed to their powerful antioxidant abilities, which may protect the body from damaging oxidation reactions, caused by 'free radicals' (Rice-Evans et al. 1997).

In this study, five 'ulam' species mainly *Anacardium occidentale, Centella asiatica, Colubrina asiatica, Pluchea indica* and *Premna cordifolia* were screened for their phenolic compounds and antioxidant activities. *Anacardium occidentale* is one of the most popular 'ulam'. The young tender leaves or the shoots of this plant are widely consumed in Malaysia, being either half-boiled or eaten fresh with rice. *Anacardium occidentale* has two varieties, i.e. red or yellow as indicated by the colour of the fruit (Maia et al. 2000). This plant is used traditionally as an anti-diabetic remedy and also claimed to show anti-protective effects in rats (Kamtchouing et al. 1998).

Centella asiatica (L.) Urban from the family of Umbelliferae (Burkhill 1966) and known locally as 'pegaga', is normally served with meals. It is a slender, green, creeping plant, rooting at the nodes. It is native to a number of countries including India, Sri Lanka, South Africa and Malaysia and used in the Ayurvedic system of medicine to treat various diseases. Reports have indicated that fresh extracts of *Centella* have been used as internal and external agents for wound healing (Kartnig 1988).

Colubrina asiatica or 'peria pantai' from the family of Rhamnaceae is found in Eastern Africa to India, Southeast Asia, tropical Australia and the Pacific Islands (Jones 1996). It is called latherleaf because of its ability to produce lather in water. It is a shrub with long, climbing or drooping branches that can reach more than 6 m in length (Jones 1996; Saidin 2000). *Colubrina asiatica* is eaten with rice after being half-boiled and it is claimed to improve digestion (Saidin 2000).

Pluchea indica is locally known as 'beluntas'. It is a shrub found widely in India and Malaysia. The height is more than one metre and it normally grows in wet-sandy soil. The leaves are obovate, green and aromatic (Saidin 2000). *Pluchea indica*

leaves and roots have been reported to possess astringent and antipyretic action. Preparations of leaves and shoots are used to treat lumbago, leucorrhoea and dysentery and as diaphoretics, nerve tonics and in poultices against atonic and gangrenous ulcer (Sen and Nag Chaudhuri 1991; Vimala et al. 2003).

Premna cordifolia is a shrub with lots of branches, silara-shaped and shady up to 2–4 m in height. Fresh young leaves are consumed with rice and have been reported to help reduce the fishy aroma of fish during cooking. The information regarding the phytochemical and biological activities of this plant is scarce. It has been claimed that aqueous extracts of leaves and roots of *P. cordifolia* can reduce fever and asthma, while the young leaves can stimulate milk production in lactating mothers (Saidin 2000).

This study was aimed at bioprospecting the 'ulam' species for their phytochemical and biological properties as the information on their chemical constituents are still lacking. The plants can be fully utilised and promoted in our local market for their nutritional values and medicinal properties.

Materials and methods

Preparation of extracts

Two batches (batch 1 and batch 2) of fresh samples of *A. occidentale* L., *C. asiatica*, *P. indica* and *P. cordifolia* were collected from Traditional Vegetables Nursery, MARDI Seberang Perai, Malaysia. *Centella asiatica* (var. Nyonya) samples were obtained from Melaka. The samples were collected during two different seasons, rainy season in September (batch 1) and dry season in February (batch 2). Fresh samples were oven-dried at temperatures below 40 ºC, weighed, ground and kept at –80 ºC prior to extraction.

Five grams of powdered samples were extracted using acidified methanol (0.1% hydrochloric acid, HCl) for one hour using shaker (IKA KS 130 basic, UK). Then the extracted samples were centrifuged (SORVALL® LEGEND RT, UK) at 3,000 g for 20 min and the supernatant was taken off and filtered using a 0.22 µm (MILLIPORE Millex GP, Ireland) before being concentrated using the rotavapor (Buchi Rotavapor R200, Japan). The extracts were weighed and dissolved in 10 ml methanol and kept at –80 ºC prior to analysis.

Determination of total phenolics using Folin-Ciocalteau assay

Two hundred microlitres of methanol extract was added to 10 ml of a 1:10 diluted Folin and Ciocalteau reagent and 1.8 ml of distilled water. After 5 min, 7 ml of Na_2CO_3 solution (115 g/litre) was added and the mixture was left at room temperature for 2 h. The absorbance of the solution was read at 765 nm against water as blank on a UNICAM UV500 (ThermoSpectronic, UK) spectrophotometer. The optical density (OD) was compared to a standard curve prepared with 50 to 500 mg/ml gallic acid and results were expressed as gallic acid equivalents (GAE).

Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay (Benzie and Strain 1996) was used in this study to estimate the antioxidant power of vegetable extracts. The method measures the ability of the extracts to reduce a ferric-2,4,6-tri-2-pyridyl-s-triazine complex (Fe^{3+} TPTZ) to the ferrous form Fe^{2+}, producing an intense blue colour with absorption at 593 nm. In the analysis, 1.5 ml of Fe-TPTZ solution was added to 50 µl of sample and 150 µl water. The absorbance at 593 nm was measured 4 min after addition of the reactant. This absorbance was compared to a 0 to 1 mM Fe^{2+} standard curve.

HPLC-diode array and MS^2 analysis

'Ulam' extracts were analysed using a Surveyor gradient HPLC system comprising of a HPLC pump, photodiode array absorbance (PDA) detector scanning from 250 to 700 nm, and an autosampler cooled

to 4 °C (Thermo Finnegan, San Jos, CA, USA). Separations were carried out using a Phenomenex (Torrance, CA, USA) (RP-MAX 4 μm 250 x 4.6 mm i.d.) C12 reverse-phase column maintained at 40 °C, eluted at 1 ml/ min with a 90 min gradient of a 10–20% gradient of acetonitrile in water containing 0.1% formic acid for *A. occidentale* and 60 min gradient of a 5–40% gradient of acetonitrile in water containing 1.0% formic acid for other species.

Separation of phenol was detected by PDA analysed at 365 nm. After the mixture passed through the flow cell of the absorbance monitor, the column eluate was split and 20% was directed to a Finnegan LCQ Advantage mass spectrometer with an electrospray interface (ESI), operating in full scan MS mode from 150 to 1000 amu. Samples were analysed using both positive and negative ionisation modes. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 400 °C. Quercetin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside and myricetin-3-*O*-rhamnoside were all quantified by reference to standard calibration curves obtained with diode array detection at λ_{max} values.

All other quercetin-derived compounds were quantified in quercetin-3-*O*-glucoside equivalents and all other kaempferol-based compounds were quantified by reference to kaempferol-3-*O*-glucoside. 5-*O*-Caffeoylquinic acid, 5-*O*-feruloyl quinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and caffeoyl feruloyl quinic acid were quantified by reference to chlorogenic acid (5-*O*-caffeoyl quinic acid) and the elution orders were verified by comparing with the coffee chlorogenic acids profiles.

Statistical analysis

All experimental results were expressed as mean values ± standard error (SD) of *n* experiments, where n = the number of samples. Analysis was carried out using either t-test or one-way analysis of variance (ANOVA). The statistical analysis was carried out using Minitab software version 12 (Minitab Inc., Addison-Wesley Publishing Co., Reading MA, USA).

Results and discussion

Total phenolic content

The levels of total phenolic content in Malaysian vegetables measured with Folin-Ciocalteau assay varied between species and between batches as shown in *Table 1*. *Anacardium occidentale* contained the highest total phenolic content compared to the other vegetables (between 1.4 and 6.2 folds in batch 1 and 2). *Centella asiatica* had the lowest total phenolic content, 100 ± 7.8 mg/kg GAE fw in batch 1 and 72 ± 0.9 mg/kg GAE fw in batch 2. In the red *A. occidentale* variety, there was no significant differences between the levels of total phenolics of the two batches. However, in the yellow variety, levels of phenolics in batch 2 (353 ± 26 mg/kg GAE fw) were significantly lower (p <0.05) than batch 1 (415 ± 20 mg/kg GAE fw). All other species showed a lower level of total phenolics in batch 2 material compared to batch 1. However, HPLC-derived phenolics did not show a similar trend to the results obtained from Folin-Ciocalteau assay as shown in *Table 1*. This could be due to some of the unknown peaks which could not be quantified due to their lower detection limit (Bloor 2001).

As shown in *Table 2*, the predominant compounds identified in *A. occidentale* were flavonols. The profile of compounds identified in the red and yellow varieties was similar in both batches. The level of flavonols in the red variety of batch 1 ranged from 20 ± 0.1 to 4308 ± 111 μg/g fw, with kaempferol-3-*O*-glucoside showing the highest concentration. In batch 2, phenolic compounds ranged from 28 ± 2.0 to 1564 ± 143 μg/g fw with quercetin-3-*O*-glucoside rather than kaempferol-3-*O*-glucoside being the predominant component. In

Table 1. Total phenolic content in selected Malaysian traditional vegetables

Traditional Vegetables	Folin-Ciocalteau method (mg/kg GAE fw)[a]		HPLC-derived phenolics (mg/g fw)[b]	
	Batch 1	Batch 2	Batch 1	Batch 2
Anacardium occidentale (Red variety)	361 ± 18	386 ± 41	12.4 ± 0.3	7.6 ± 0.7*
Anacardium occidentale (Yellow variety)	415 ± 20	353 ± 26*	6.4 ± 0.4	8.5 ± 0.2*
Centella asiatica	100 ± 7.8	72 ± 0.9*	3.5 ± 0.2	3.2 ± 0.9*
Colubrina asiatica	105 ± 5.6	62 ± 2.5*	0.9 ± 0.0	0.7 ± 0.0*
Pluchea indica A	294 ± 39	97 ± 2.7*	2.5 ± 0.0	1.6 ± 0.0*
Pluchea indica B	316 ± 18	135 ± 8.9*	9.7 ± 0.2	2.3 ± 0.1*
Premna cordifolia	187 ± 1.0	170 ± 9.1*	4.4 ± 0.0	8.3 ± 0.5*

[a]Total phenolic content analysed by Folin-Ciocalteau method
[b]Accumulation of individual phenolics analysed by HPLC-PDA-MS^2
*Indicates significantly different (p <0.05) between batches; n = 6

Table 2. Individual phenolics in two batches of *Anacardium occidentale* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (μg/g fw)			
	var. red		var. yellow	
	Batch 1	Batch 2	Batch 1	Batch 2
Myricetin glycoside	65 ± 0.5	212 ± 18	61 ± 4.4	160 ± 2.3
Myricetin glycoside	75 ± 2.4	148 ± 14	46 ± 4.1	173 ± 3.9
Unknown quercetin conjugate*	20 ± 0.1	28 ± 2.0	12 ± 0.8	35 ± 0.8
Unknown quercetin conjugate*	77 ± 3.4	664 ± 84	125 ± 2.5	637 ± 34
Myricetin-3-*O*-rhamnoside	25 ± 0.5	59 ± 3.4	nd	79 ± 0.6
Quercetin-3-*O*-galactoside	771 ± 9.3	1321 ± 122	600 ± 32	1503 ± 23
Quercetin-3-*O*-glucoside	829 ± 5.8	1564 ± 143	663 ± 39	1672 ± 25
Quercetin-3-*O*-xyloside	566 ± 4.5	1034 ± 90	481 ± 20	1149 ± 18
Quercetin-3-*O*-arabinofuranoside	459 ± 7.7	718 ± 48	338 ± 11	897 ± 11
Quercetin-3-*O*-arabinopyranoside	109 ± 2.5	597 ± 43	60 ± 0.2	822 ± 23
Quercetin-3-*O*-rhamnoside	481 ± 13	892 ± 77	374 ± 21	967 ± 16
Kaempferol-3-*O*-glucoside	4308 ± 111	145 ± 7.3	2592 ± 160	171 ± 3.1
Kaempferol-3-*O*-xyloside	388 ± 27	41 ± 1.5	257 ± 22	48 ± 0.4
Kaempferol-3-*O*-arabinofuranoside	633 ± 46	71 ± 2.6	477 ± 43	96 ± 0.4
Kaempferol-3-*O*-arabinopyranoside	3307 ± 39	86 ± 3.7	350 ± 53	101 ± 1.5
Kaempferol coumaroyl glucoside	310 ± 30	nd	nd	nd
HPLC-derived total phenolics	12423 ± 303	7580 ± 660	6436 ± 413	8510 ± 163

nd = not detected
*Unknown quercetin conjugates were quantified based on quercetin's standard calibration curve; n = 6

batch 1 of the yellow variety, kaempferol-3-*O*-glucoside also showed the highest concentration, and the concentration of phenolics ranged from 12 ± 0.8 to 2592 ± 160 μg/g fw. Similarly, in batch 2, level of quercetin-3-*O*-glucoside was the highest and the phenolic concentrations ranged from 35 ± 0.8 to 1672 ± 25 μg/g fw. The total phenolics derived from the HPLC-PDA-MS^2 analysis varied and were significantly different (p <0.05) between species, which showed that total phenolics was the highest in batch 1 of the red variety. In contrast, the level of phenolics in batch 2 was the highest in the yellow variety. This was also in contrast to the total phenolic content analysed by Folin-Ciocalteau method.

In batch 1 of *C. asiatica*, chlorogenic acids were present in substantial amounts, but flavonols were the major components

Table 3. Individual phenolics in two batches of *Centella asiatica* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (μg/g fw)	
	Batch 1	Batch 2
Quercetin-3-*O*-glucoronide	55 ± 1.1	16 ± 0.7
Kaempferol-3-*O*-glucoside	526 ± 34	493 ± 8.3
Kaempferol-3-*O*-glucuronide	1633 ± 145	nd
Total flavonol	2214 ± 180	509 ± 9.0
3-*O*-Caffeoylquinic acid	14 ± 0.5	27 ± 5.2
5-*O*-Caffeoylquinic acid	86 ± 1.6	113 ± 5.5
5-*O*-Feruloylquinic acid	18 ± 0.5	47 ± 1.2
3,4-*O*-Dicaffeoylquinic acid	408 ± 4.2	126 ± 1.7
3,5-*O*-Dicaffeoylquinic acid	671 ± 16	718 ± 30
4,5-*O*-Dicaffeoylquinic acid	nd	163 ± 4.7
3-*O*-Feruloyl-5-*O*-caffeoylquinic acid	41 ± 1.2	1229 ± 24
3-*O*-Caffeoyl-5-*O*-feruloylquinic acid	72 ± 1.0	106 ± 10
Total chlorogenic acids	1310 ± 25	2529 ± 82
HPLC-derived total phenolics	3524 ± 205	3038 ± 91

nd = not detected; (n = 6)

Table 4. Individual phenolics in two batches of *Colubrina asiatica* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (μg/g fw)	
	Batch 1	Batch 2
Quercetin-3-*O*-rhamnoside	398 ± 2.3	242 ± 17
Kaempferol-3-*O*-glucoside	103 ± 1.5	29 ± 2.0
Kaempferol-3-*O*-rutinoside	371 ± 1.7	392 ± 18
HPLC-derived total phenolics	872 ± 5.5	663 ± 37

n = 6

present (63% of the total phenolics, ranging from 55 ± 1.1 to 1633 ± 145 μg/g fw) (*Table 3*). However, in contrast, in batch 2, chlorogenic acids were predominant ranging from 27 ± 5.2 to 1229 ± 24 μg/g fw (84% of the total phenolics).

In *C. asiatica*, only three phenolic compounds were identified and quantified (*Table 4*). In batch 1, levels of individual phenolics ranged from 103 ± 1.5 to 398 ± 2.3 μg/g fw where quercetin-3-*O*-rhamnoside was the major compound. In batch 2, kaempferol-3-*O*-rutinoside was the main constituent. The HPLC-derived total phenolics of this plant was the lowest (0.9 mg/g fw in batch 1 and 0.7 mg/g fw in batch 2) among the species investigated. Batch 1 contained more phenolics than batch 2. This is in agreement with the total phenolic content analysed by Folin-Ciocalteau method.

Pluchea indica grown under different environments (var. A, under shade and var. B, exposed to sunlight) showed different profiles of compounds and levels of individual phenolics varied (*Table 5*). Chlorogenic acids were the major constituents (91% of the HPLC-derived total phenolics) with 3,5-*O*-dicaffeoylquinic acid predominantly in var. A of batch 1. The level of total phenolics in batch 2 was lower than that of batch 1 in var. A. However, the level of total phenolics analysed by HPLC-PDA-MS^2 was significantly (p <0.05) higher in batch 1 of var. B (9723 ± 151 μg/g fw) compared to other variety of both batches.

Table 5. Individual phenolics in two batches of *Pluchea indica* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (μg/g fw)			
	var. A		var. B	
	Batch 1	Batch 2	Batch 1	Batch 2
Quercetin-3-*O*-galactoside	5.8 ± 0.8	nd	47 ± 0.3	nd
Quercetin-3-*O*-glucoside	29 ± 0.3	nd	134 ± 2.8	nd
Quercetin-3-*O*-sulphate	187 ± 6.6	303 ± 5.5	1496 ± 17	35 ± 1.1
Total flavonol	222 ± 7.7	303 ± 5.5	1677 ± 20.1	35 ± 1.1
3-*O*-Caffeoylquinic acid	13 ± 0.4	nd	181 ± 0.5	nd
5-*O*-Caffeoylquinic acid	132 ± 0.1	9.8 ± 0.2	881 ± 4.2	48 ± 2.3
3,4-*O*-Dicaffeoylquinic acid	131 ± 3.7	662 ± 21	573 ± 4.8	676 ± 25
3,5-*O*-Dicaffeoylquinic acid	1011 ± 27	586 ± 7.8	3128 ± 52	1361 ± 31
4,5-*O*-Dicaffeoylquinic acid	958 ± 7.0	nd	3214 ± 67	nd
Tricaffeoylquinic acid	68 ± 0.8	68 ± 4.6	69 ± 2.1	164 ± 16
Total chlorogenic acids	2313 ± 39	1326 ± 34	8046 ± 131	2249 ± 74
HPLC-derived total phenolics	2535 ± 47	1629 ± 39	9723 ± 151	2284 ± 75

nd = not detected; n = 6

Table 6. Individual phenolics in two batches of *Premna cordifolia* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (μg/g fw)	
	Batch 1	Batch 2
Isorhamnetin	nd	93 ± 5.4
Methylquercetin glycoside conjugate*	3046 ± 30	nd
Methylquercetin glycoside conjugate*	341 ± 1.5	5959 ± 37
Total flavonol	3387 ± 32	6052 ± 42
Apigenin-7-*O*-rutinoside	997 ± 10	2287 ± 434
HPLC-derived total phenolics	4384 ± 42	8339 ± 476

*Quantified based on quercetin standard
nd = not detected; n = 6

Quercetin glycosides were not identified in batch 2 of both varieties indicating the different effect of season to the metabolism of plant metabolites. 3-*O*-caffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid were also not present in batch 2 of both varieties.

Four phenolic compounds were identified in *P. cordifolia* (*Table 6*) in concentrations ranging from 341 ± 1.5 to 3046 ± 30 μg/g fw in batch 1 and from 93 ± 5.4 to 5959 ± 37 μg/g fw in batch 2. Flavonols were the predominant compounds (77% in batch 1 and 73% in batch 2 from the total individual phenolics). One flavone, apigenin-7-*O*-rutinoside was identified and quantified. The level of HPLC-derived total phenolics in batch 2 was significantly higher than that of batch 1. Isorhamnethin was present only in batch 2. In batch 1, two types of methylquercetin glycoside conjugates were detected and quantified based on quercetin standard.

Correlation between total phenolic content and total antioxidant activities

The total phenolic content of Malaysian traditional vegetables has been indicated to be correlated by the contribution of individual phenolic compounds. The total antioxidant activities measured by FRAP assay are shown in *Figure 1*. Among the species, *A. occidentale* showed the highest total antioxidant activity in both batches 1 and 2 up to 4.5 folds compared to the

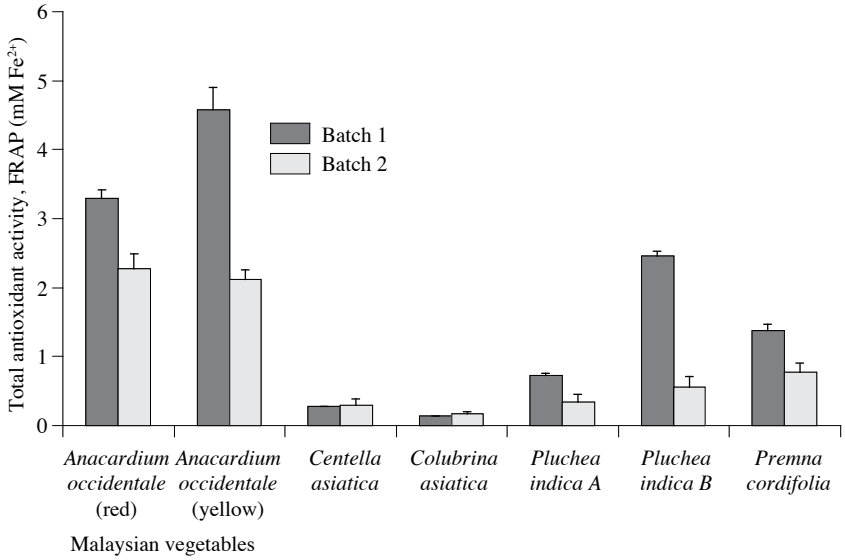

Figure 1. Total antioxidant activity (FRAP) of Malaysian traditional vegetables. Results expressed as value ± standard deviation, SD (n = 6)

Table 7. Pearson correlation of total phenolic content and total antioxidant activities (FRAP) of traditional vegetables (a = p <0.001, b = p <0.005)

	Anacardium occidentale		*Centella asiatica*	*Colubrina asiatica*	*Pluchea indica*		*Premna cordifolia*
	Red	Yellow			A	B	
Batch 1	0.987a	0.602	–0.614	–0.950a	0.851b	0.101	–1.000a
Batch 2	0.974a	0.946a	0.267	0.945a	0.921a	0.149	0.968a

other species. Of the two varieties, the yellow one had the highest activity (4.6 ± 0.3 mM in batch 1 and 2.1 ± 0.1 mM in batch 2). This was correlated to the total phenolic content, which showed the highest concentration among the vegetables tested. *Centella asiatica* exhibited the lowest total antioxidant activity for both batches (0.1 ± 0 mM in batch 1 and 0.2 ± 0 mM in batch 2). The rank of these vegetables based on FRAP-derived antioxidant activity was *A. occidentale* (yellow) > *A. occidentale* (red) > *P. indica* (var B) > *P. cordifolia* > *P. indica* (var A) > *C. asiatica* > *C. asiatica*.

There were significant correlations between the total phenolic content and the total antioxidant activities in FRAP assay. For example, in the red variety of *A. occidentale,* FRAP-derived antioxidant activity was highly and significantly correlated with total phenolic content, r = 0.987 (p <0.001) in batch 1 and r = 0.974 (p <0.001) in batch 2 (*Table 7*). Tsao and Deng (2004) also reported that high antioxidant activity was correlated with total phenolic content. This was supported by the fact that phenolic compounds are more effective antioxidants than other compounds such as vitamin C and E in fruits and vegetables which contribute to the protective effects mechanism in humans (Rice-Evans et al. 1997). Other studies, however, reported that there was no correlation between total phenolic content and the total antioxidant activities of 92 species of fruits and vegetables analysed by Folin-Ciocalteau for total phenolic content and oxidised Melo method for *in vitro* antioxidant

activity (Kahkonen et al. 1999). Therefore, determination of phenolic compounds and their contribution to the total antioxidant activity are required to understand the correlation between the phenolic compounds and the antioxidant activities in fruits and vegetables. However, direct comparison could not be made as the samples measured in the studies were different from the 'ulam'extracts used in these present study. In addition, the method used to analyse the samples was also different which also influenced the outcome (Puupponen-Pimia et al. 2001).

High levels of total phenolics were observed in all species of 'ulam' in the present study and the amount varied greatly between the two batches of harvesting. The total phenolic content was statistically lower (p <0.05) in batch 2, which was harvested in the dry season compared to batch 1 which was obtained in the rainy season. This could be due to the complex metabolism of secondary metabolites in plants that may be affected by various factors such as light intensity, humidity and different harvesting periods. For instance, the increase in total phenolic content during the rainy season (batch 1) could be due to phenolic esterification as reported by Solecta et al. (1999).

Acclimatisation, especially low temperature and high humidity, are reported to influence the modification of metabolic pathways in plants (Kacperska 1989) especially phenylpropanoid metabolism (Dixon and Paiva 1995). The key enzyme of phenylpropanoid biosynthesis, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is reported to increase in tomatoes (Rhodes and Wooltorton 1977; Fenn 1994) and potatoes (Tanaka and Uritani 1977; Fenn 1994) after the temperature is reduced by chilling. Chalker-Scott and Fuchigami (1989) also reported accumulation of water-soluble phenolics in frost-stressed rhododendron leaves and there was increased lignification of grapevine, apple trees and sugar cane subjected to low temperature.

In the present study, even though there was variability of total phenolic content between species, identification of individual phenolics has revealed similar type of flavonoid compounds, that could also contribute to the amount of total phenolic content. HPLC-derived total phenolic content was lower compared to total phenolics content analysed by Folin-Ciocalteau assay but in line with the previous observation i.e. batch 1 had a higher phenolic content compared to that of batch 2. The lower values of the HPLC-derived total phenolic content and some variations between the results of batch 1 and batch 2 could be due to the unknown phenolic compounds that could not be quantified and high molecular weight compounds, such as polymeric procyanidins, that did not elute from the HPLC column (Robards 2003). Furthermore, some of the flavonoid conjugates were quantified on the basis of their parent aglycones as no standards were available, for example, methylquercetin glucoside conjugate was quantified by reference to a quercetin standard curve. The level of total phenolics in the traditional vegetables can be ranked in the following order: *A. occidentale* > *P. indica* > *P. cordifolia* > *C. asiatica* > *C. asiatica*.

Among the flavonoid groups, flavonol glycosides were shown to be predominant in most of the species particularly in *A. occidentale, C. asiatica* and *P. cordifolia*. The flavonols identified in the present study were similar to previous studies on other vegetables (Justesen et al. 1998; Crozier et al. 2000). Other flavonoid glycosides are also reported to be predominant forms of naturally occurring flavonoids in plants (Cuyckens et al. 2000), which represent a large group of secondary plant metabolites (Harborne et al. 1988).

Analysing these compounds by HPLC-MS^2 has facilitated identification based on mass spectra fragmentation pattern; however, there are limitations when it comes to identifying compounds with a

similar molecular weight. Therefore, the need to compare absorbance spectra and retention times (elution order) with available standards which helps to verify the identity of compounds. Bloor (2001) has discussed general rules of reverse-phase HPLC elution order to confirm identities of flavonol glycosides. The order of elution from most polar to least polar means that flavonol triglycosides (and higher glycosides) elute early, followed by di- and monoglycosides and then acylated or alkylated glycosides and aglycones.

Hydrolysis with HCl can also be proposed for the analysis of flavonoids where sugar moieties are cleaved releasing the aglycone (Hertog et al. 1992). However, as most of the flavonoids detected in these traditional vegetables were flavonols, in particular quercetin- and kaempferol-based compounds, which are reported to be predominant in plants, the need for acid hydrolysis was not necessary as this technique will remove the possibility to identify the conjugation of the compound. This is in agreement with previous reports (Harborne et al. 1988; Heim et al. 2002). There is increasing interest not only in phytochemical identification, but also in the bioactivities of these compounds *in vitro* and *in vivo* especially with regard to antioxidant and anticancer properties. The present study indicated that quercetin and kaempferol are among the most important flavonoids that could contribute to such bioactivities.

Chlorogenic acids are characteristic components of coffee beans and commercial coffee products, in which caffeoylquinic, *p*-coumaroylquinic, feruloylquinic, dicaffeoylquinic and caffeoylferuloylquinic acids were reported to possess high antioxidant activity (Clifford 2000; Clifford et al. 2003). In the present study, chlorogenic acids were the major components found in *C. asiatica* and *P. indica*. Identification of these compounds was based on the MS^2 fragmentation patterns (Clifford et al. 2003) and comparison of the elution order of chlorogenic acids found in coffee analysed under similar HPLC conditions.

The level of total chlorogenic acids in *C. asiatica* was found to be higher in batch 2 compared to batch 1 by two-fold. This is in contrast with *P. indica*, where the level of total chlorogenic acids was higher in batch 1 (var. A by 2-fold and var. B by 4-fold). However, the level of total chlorogenic acids in var. B, which was grown under direct sunlight, was approximately 4-fold higher than var. A, which was grown under shade. The effect of sunlight on the production of chlorogenic acids in plant has been previously studied (Zucker 1963; Vicente et al. 2009). When longer light exposures at higher intensities are given, chlorogenic acid synthesis is stimulated, and light appears to enhance chlorogenic acid formation by virtue of its effects on protein synthesis (Zucker 1963, Vicente et al. 2009). Levels of different chlorogenic acids also varied greatly in these two species which arguably indicates an internal factor such as the metabolism of chlorogenic acid (5-caffeoylquinic acid) to dicaffeoyl- and tricaffeoylquinic acids (Clifford 2000). Genetic engineering has also increased chlorogenic acid levels in plants such as tomato and tobacco, in order to increase antioxidant potential for health benefits (Niggeweg et al. 2004).

As other dietary polyphenols, chlorogenic acid is an antioxidant, which has been reported to scavenge radicals generated in the aqueous phase *in vitro*, increase the resistance of LDL to lipid peroxidation and inhibit DNA damage (Kasai et al. 2000). Apart from antioxidant activity *in vitro*, chlorogenic acids when added to the diet treatment *in vivo*, inhibited chemically induced carcinogenesis of the large intestine, liver and tongue in rats and hamsters (Tanaka et al. 1993) and therefore, could be a potential anticancer agent.

There were correlations between total phenolic content and FRAP-derived total antioxidant activities in these plants (*Table 7*). Significant correlations

between total phenolic content and total antioxidant activities in batch 2 of all the species were observed in the present study, but no significant correlations ($p > 0.005$) were observed in *C. asiatica, C. asiatica* and *P. cordifolia* in batch 1. The discrepancy could be due to the lower number of samples in the analysis. Many reports have indicated a correlation between total phenolic content and total antioxidant activities *in vitro* in vegetables, and phenolics have been attributed to be the main contributor for these biological activities (Proteggente et al. 2003; Ismail et al. 2004). However, several reports did not observe any correlations between total phenolic content and total antioxidant activities (Kahkonen et al. 1999; Puupponen-Pimia et al. 2001). The reason was due to different phenolic compounds giving different responses in the Folin-Ciocalteau method (Satue-Gracia et al. 1997) and may be a consequence of fruits and vegetables containing other phytochemicals such as vitamins and alkaloids that were reported to exhibit potent antioxidant activity (Rice-Evans and Miller 1995; Heim et al. 2002).

Anacardium occidentale was shown to have the highest antioxidant activity in all the assays tested and in line with the Folin-Ciocalteau assay. This plant is popular in Malaysia, and the highest antioxidant activity exhibited by *A. occidentale* is in agreement with previous reports (Vimala et al. 2003; Abas et al. 2006). However, these earlier studies used thiobarbituric acid (TBA), ferric thiocyanate (FTC) and DPPH assays, which were different methods to that of the present study. Maia et al. (2000) reported that *A. occidentale* also contained other compounds that could contribute to the antioxidant activity, such as, anacardic acids which were found to be abundant in fruits of *A. occidentale* (Kubo et al. 2006; Schultz et al. 2006).

Surprisingly, *C. asiatica,* which is also a very popular traditional vegetable in Malaysia and reported to have high antioxidant activity (Vimala et al. 2003; Hussin et al. 2007) and the ability to increase the levels of antioxidant enzymes such as SOD, glutathione peroxidase and GSH significantly in rats *in vivo* (Veerendra Kumar and Gupta 2002), showed the lowest antioxidant activities among the vegetables evaluated in the present study. This, however, could be influenced by several factors such as different samples used, different types of extracts (water, methanol or chloroform extracts), methodology used and also the effects of environmental factors such as different harvesting season, sunlight and storage (Harborne and Williams 2000; Robards 2003).

Total antioxidant activities exhibited by phenolic compounds in fruits and vegetables, as stated, were correlated to the protective effects, which reduced the risk of chronic diseases in man (Arts and Hollman 2005). The antioxidant activities of phenolic compounds are closely related to their structures. The common flavonoids such as myricetin, quercetin and rutin were shown to exert greater antioxidant activities than the conventional antioxidant vitamin, α-tocopherol (vitamin E) but the bioavailability may be much less (Afanas'ev et al. 1989; Rice-Evans et al. 1996). This is due to the number of hydroxyl groups which enable the compound to donate H^+ and delocalise the resulting free electron (Awad et al. 2001; Heim et al. 2002). The more hydroxyl groups attached in a molecule, the greater the antioxidant activitiy (Salah et al. 1995). Thus, similar amounts of quercetin and myricetin have very high TEAC values, 4.7 and 3.1 mM respectively (Rice-Evans et al. 1996).

In the present study, *A. occidentale* exhibited very high antioxidant activities, which is probably due to the presence of myricetin and quercetin glycosides. However, it was reported that the aglycones are more potent antioxidants than their corresponding glycosides as the attached glucose lower the radical scavenging activity (Heim et al. 2002). Nevertheless, synergistic effects from other compounds

present in plants can also influence the total antioxidant activity (Liu 2003). In the present study, the total aglycones were not quantified, and flavonoid glycosides were the predominant components in the plants under study.

Conclusion

The red and yellow varieties of *A. occidentale* contained high amounts of phenolic compounds. This plant also showed very high total antioxidant activities which could contribute to lower the risk of chronic diseases. However, *C. asiatica* has low phenolics and total antioxidant activities but comprises different types of phenolic compounds (including chlorogenic acids) instead of flavonol glycosides found in *A. occidentale*. All the 'ulam' species are good sources of beneficial and antioxidant compounds. This study has shown the potential of the 'ulam' species to be fully utilised and consumed by our local people.

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Abstrak

Pengambilan buah-buahan dan sayuran segar dalam diet harian dapat mengurangkan risiko mendapat penyakit berbahaya seperti kardiovaskular dan kanser. Buah-buahan dan sayuran segar juga dilaporkan secara meluas menjadi sumber bahan antioksida. Lima jenis ulam, iaitu *Centella asiatica* (L.) Urban (pegaga), *Anacardium occidentale* L. (gajus), *Colubrina asiatica* (peria pantai), *Pluchea indica* (beluntas) dan *Premna cordifolia* (bebuas) dari janaplasma MARDI, Sebarang Perai telah disaring kandungan sebatian fenol dan dianalisis aktiviti antioksida menggunakan asai FRAP (Ferric Reducing Antioxidant Potential). Jumlah kandungan fenol bagi sayuran ini antara 100–415 mg/kg bersamaan asid galik (GAE) pada musim pertama dan antara 62–386 mg/kg GAE pada musim kedua. *Anacardium occidentale* mempunyai jumlah kandungan fenol yang paling tinggi diikuti oleh *P. indica* B dan A, *P. cordifolia, C. asiatica* dan yang paling rendah *C. asiatica*. Bagi aktiviti antioksida, jumlah paling tinggi dipamerkan oleh *A. occidentale* yang berkemungkinan disebabkan oleh kandungan fenol yang tinggi. Flavonol glikosida paling dominan dalam kebanyakan spesies ulam terutamanya *A. occidentale* dengan kandungan sebanyak 6.4–12.4 mg/g berat basah. Asid klorogenik ialah komponen utama yang dikenal pasti dalam *C. asiatica* dan *P. indica*. Dalam kajian ini, jumlah kandungan fenol didapati mempunyai korelasi positif dengan aktiviti antioksida di dalam ekstrak ulam.

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