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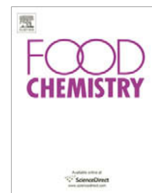

Assessment of *in vitro* antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia

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ABSTRACT

Determination of total phenolic (TPC), flavonoid and anthocyanin contents, and various antioxidant activities (2,2-diphenyl-1-picrylhydrazyl radical scavenging, ferric reducing power, ferrous ion chelating and lipid peroxidation inhibition) of leaves and flowers of *Bauhinia kockiana*, *Caesalpinia pulcherrima* and *Cassia surattensis* were performed in this study. The *B. kockiana* flower was found to possess the highest TPC (8280 ± 498 mg GAE/100 g), free radical scavenging activity (ascorbic acid equivalents 14,600 ± 2360 mg AA/100 g) and reducing ability (72.4 ± 8.7 mg GAE/g). Rutin and chlorogenic acid were detected in the plants, where the *C. pulcherrima* leaf contained the highest amount of rutin (669 ± 26 mg/100 g), while minute amounts of chlorogenic acid were detected in *C. surattensis* leaf (9.13 ± 0.44 mg/100 g). The *C. pulcherrima* leaf displayed the highest ferrous ion chelating and lipid peroxidation inhibition activities. Positive correlation was observed between TPC and various antioxidant activities.

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1. Introduction

An enormous variety of secondary metabolites such as alkaloid, terpenes, saponines, quinones and polyphenols are synthesised exclusively by plants for various purposes, i.e. as a chemical defense against herbivores and microbial attack (Wink, 1988), toxicant and repellent for insects, and attractants for pollinators. Close and McArthur (2002) suggested that polyphenols, including the low molecular weight flavonoids produced by plants, are primarily for protection against photodamage. Plant polyphenols with antioxidant capacity could scavenge reactive chemical species as well as to minimise oxidative pressure resulting from excessive light energy exposure. Some plant polyphenols are important components of both human and animal diets and they are safe to be consumed. These polyphenols could function as metal catalyst chelators, inhibit LDL peroxidation, and aid in minimising tissue oxidative damages as well as free radicals released during various biochemical processes in a living organism's body (Gülçin, Berashvili, & Gepdiremen, 2005).

Many plants from the Leguminosae family are medicinal herbs which are easily found in Malaysia. They are grown as weeds (i.e. *Mimosa pudica*), woody shrubs (*Peltophorum pterocarpum*), crops (*Arachis hypogaea*) and vines (*Bauhinia kockiana*). Some of these plants are edible, and hence they are utilised for various purposes in food, beverages, and food colouring agent (Goh, 2004; Ong, 2006). These plants are commonly used as traditional medicines to treat various health complications. In our present study, the antioxidant properties of three species from the Leguminosae family, namely *B. kockiana* Korth, *Caesalpinia pulcherrima* (L) Sw. and *Cassia surattensis* Burm. F. are examined and this is the first report on the *in vitro* assessment on their antioxidant activity.

B. kockiana is a tropical vine which is also cultivated as a garden ornamental plant because of its bright orange–red magnificent inflorescences. This plant originates from the Malaysia tropical forest and its roots are used by the *Kelabit* ethnic group in Sarawak to treat gonorrhoea (Fasihuddin, Ipor, & Din, 1995). Besides, the infusion of the roots is consumed orally to treat nervous debility, insomnia and fatigue. The bark and root are also used traditionally to treat toothache (Ong, 2006).

C. pulcherrima or commonly known as peacock flower consists of flowers with bright red petals. It is a shrub growing to 6 m tall, native to tropical America (Goh, 2004) but now it is widely grown in both tropical and subtropical area. The infusion of the flowers is used to cure bad cough, asthma and sores. Traditionally, the leaves decoction is consumed orally to cure fever and constipation.

C. surattensis, also known locally as bushy cassia is a flowering plant which is easily seen along the roadside of Malaysia. It is a shrub growing to 6 m tall, with many branches. The leaves are bipinnate, each bearing 14–18 leaflets, 25 mm long and 12 mm broad (Goh, 2004). The leaves are boiled and the infusion is consumed orally to cure sore throat, cough and constipation by the Chinese.

The objectives of this study were to evaluate the antioxidant capacity by spectrophotometric determination of total phenolic content (TPC), total flavonoid content (TFC), total anthocyanins

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content (TAC), free radical scavenging ability (via DPPH radical scavenging assay), ferric reducing power (FRP), ferrous ion chelating activity (FIC) and lipid peroxidation inhibition effect (via BCB assay), and to determine polyphenol compositions of extracts using high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Chemicals

Folin–Ciocalteu's phenol reagent, gallic acid, trifluoroacetic acid, linoleic acid and Tween 40 were purchased from Fluka (Switzerland). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, β-carotene, rutin, quercetin and chlorogenic acid were obtained from Sigma (St. Loius, MO). L(+) ascorbic acid was purchased from Merck (Germany). Ferrozine was purchased from Acros Organics (Pittsburgh, PA). All other common chemicals were obtained from Fisher Chemicals (Springfield, NJ) and Merck. Solvent used for HPLC analysis was of HPLC grade, while all other organic solvents were of analytical grade. All organic solvents were obtained from Fisher Chemicals. Water used was of Millipore quality.

2.2. Plant materials

B. kockiana, *C. pulcherrima* and *C. surattensis* flowers and leaves were collected from Klang Valley in Peninsular Malaysia and were identified by Mr. Anthonysamy Savarimuthu. The plant materials were collected on the day when extraction was performed. Voucher specimens (MUM-LEGUM-001 – MUM-LEGUM-003) were deposited in the herbarium of School of Science, Monash University Sunway campus.

2.3. Preparation of extracts

Flowers and leaves (1 g) of each were frozen with liquid nitrogen and were ground manually in a mortar. The powdered materials were then extracted with 75% v/v methanol (50 ml), with continuous shaking with an orbital shaker (Protech, Model 719) for an hour at room temperature. The extracts were filtered using a vacuum pump aspirator (A-35 Eyela Tokyo Rikakikai Co. Ltd., Japan) and kept at −20 °C until further analysis was performed.

Extraction efficiency of each sample was determined by three times extractions. The residues which were obtained after the filtration were transferred back into the extraction flask, followed by addition of 50 ml fresh 75% methanol.

2.4. Total phenolic content (TPC)

The total phenolic content (TPC) of extracts was measured using the Folin–Ciocalteu method as described by Chew, Lim, Omar, and Khoo (2008). Folin–Ciocalteu's phenol reagent (1.5 ml, 10% v/v) and 1.2 ml 7.5% w/v Na_2CO_3 were added to the 0.3 ml sample extract. The reaction mixture was thoroughly mixed and was incubated in the dark for 30 min. The absorbance of the reaction mixture was then measured at 765 nm (spectrophotometer U-1800 Hitachi, Japan), and TPC was expressed in terms of mg gallic acid equivalents (GAE) per 100 g fresh material. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$), where x is the gallic acid concentration in mg/L and y is the absorbance reading at 765 nm.

2.5. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging assay was measured based on the method used by Leong and Shui (2002). Two millilitre of 0.15 mM DPPH (in methanol) was added to the different dilutions of the extract (amounting to 1.0 ml). The reaction mixture was incubated for 30 min after which its absorbance was measured at 517 nm, where methanol was used as both a blank and negative control. The decrease in absorbance was calculated as an IC_{50} and expressed as mg ascorbic acid (AA) equivalents per 100 g of fresh material (AEAC) as follows:

$$\mathrm{AEAC(mg\ AA/100\ g)} = \mathrm{IC_{50(ascorbate)}}/\mathrm{IC_{50(sample)}} \times 10^5$$

$IC_{50(ascorbate)}$ of 0.00382 mg/ml was used in AEAC calculation.

2.6. Ferric reducing power (FRP)

The ferric reducing power (FRP) described by Chu, Chang, and Hsu (2000) was adopted. Potassium phosphate buffer (2.5 ml of 0.1 M and pH 6.6) and 2.5 ml of 1% w/v potassium ferricyanide were mixed with 1.0 ml of extracts of varying dilutions. The reaction mixture was incubated at 50 °C for 20 min, after which 2.5 ml of 10% w/v trichloroacetic acid was added. Water (2.5 ml) and 0.5 ml of 0.1% w/v $FeCl_3$ were then added to 2.5 ml of the reaction mixture, and the solution was incubated at 28 °C for 30 min to facilitate colour development. The absorbance was measured at 700 nm and the amount of gallic acid equivalents in mg per gram fresh material (mg GAE/g) was calculated. The calibration equation for gallic acid was $y = 16.21x - 0.0206$ ($R^2 = 0.9970$), where x is the gallic acid concentration in mg/ml, and y is the absorbance reading at 700 nm.

2.7. Determination of total anthocyanins (TAC)

The total anthocyanins (TAC) were determined by the pH differential method, as described by Moyer, Hummer, Finn, Frei, and Wrodstad (2002). One millilitre of 0.2 M potassium chloride solution (adjusted to pH 1.0 with 1.0 M HCl) and 1 M sodium acetate buffer (adjusted to pH 4.5 with 1.0 M HCl) were added into 2.0 ml of extracts respectively. The absorbance was then measured at 520 and 700 nm. The absorbance at 700 nm was subtracted from the absorbance at 520 nm. The concentration of anthocyanins was calculated as follows:

$$\text{Concentration of anthocyanins (mg/L)} = A/\varepsilon L \times \mathrm{MW} \times 10^3 \times \text{Dilution Factor}$$

where A is the difference of absorbance between pH 1.0 and 4.5; ε is molar extinction coefficient for cyanidin-3-glucoside (26,900); L is the path length of the spectrophotometer cell (1.0 cm), and MW is molecular weight of cyanidin-3-glucoside (449.2 g/mol). The result was expressed as the amount of cyanidin-3-glucoside (cy-3-glu) equivalents per 100 g of sample (mg cy-3-glu/100 g sample).

2.8. Total flavonoid content (TFC)

Total flavonoid content (TFC) was assayed as described by Chang, Yang, Wen, and Chern (2002). In brief, extract (0.5 ml) was mixed with 75% methanol (1.5 ml), 10% w/v aluminium chloride (0.1 ml) and 1.0 M potassium acetate (0.1 ml) and water (2.8 ml). The reaction mixture was allowed to incubate for 30 min at room temperature before the absorbance was taken at 435 nm. Water (0.1 ml) was used to substitute 10% aluminium chloride for blank.

Rutin was used as a standard for the calibration, and a calibration curve (ranging from 20 to 40 mg/L) was generated, with the regression equation of $y = 0.019x$ ($R^2 = 0.9980$), where x is the concentration of rutin in mg/L, and y is the absorbance reading at 435 nm. The result was expressed as mg rutin equivalents per 100 g fresh material (mg RE/100 g).

2.9. HPLC analysis

Analytical chromatographic analysis was performed using liquid chromatography Agilent Technologies series 1200 (Germany), which consisted of a G1311A quaternary pump, G1315B diode array detector, G1322A vacuum degasser and was equipped with a reversed-phase column, Eclipse XDB-C18 (150×4.6 mm) with 5 μm particle size (Agilent, USA). Acetonitrile in water (5% and 40%) acidified to pH 2.5 with trifluoroacetic acid were used as the mobile phase. The solvent gradient was performed as follows: 5–40% acetonitrile in a linear gradient, from 0 to 45 min, followed by 40% acetonitrile in isocratic mode, from 46 to 60 min. The flow rate was fixed at 1 ml/min.

All samples and standards were prepared in 75% methanol and were filtered through a 0.45 μm membrane filter (Millipore, USA) prior to injection. Two-hundred microlitre of the standards and samples were injected manually. The chromatogram was obtained at 365 nm, with a reference wavelength of 600 nm. The spectra were acquired from 200 to 700 nm.

2.9.1. Identification and quantification of polyphenolic compounds

Polyphenolic compounds present in samples were identified by comparing their retention times with standard commercial polyphenolic compounds. Confirmation was performed by co-injection, and comparing the ultraviolet (UV) spectra of the samples and standards.

For each standard, 1 mg/ml of stock solution was prepared. Various concentrations of standard solutions for the calibration were prepared by diluting the stock solutions. A calibration curve was plotted using peak areas from chromatograms against a known concentration of standards, and the best fit line equation for the respective standard was generated.

2.10. Ferrous ion chelating (FIC) assay

The ferrous ion chelating (FIC) assay was adapted from a method described by Gülçin (2005). Briefly, 0.1 mM $FeSO_4$ (1 ml) was added to various dilutions of the extracts (1 ml; 1.0–7.0 mg/ml), followed by 0.25 mM ferrozine (1 ml). The reaction mixtures were allowed to stand for 10 min before the absorbance measurements were taken at 562 nm. The ferrous ion chelating property of extracts was calculated as a percentage using the following formula:

$$\text{Chelating activity } (\%) = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$$

where A_{control} and A_{sample} are the absorbance of the control and extract, respectively. The control contained 1 ml each of 75% methanol, $FeSO_4$ and ferrozine.

2.11. β-Carotene bleaching (BCB) assay

A modified method of the β-carotene bleaching (BCB) assay described by Lim and Quah (2007) was employed in this study. Three millilitre of β-carotene (5 mg/50 ml in chloroform) was added to linoleic acid (40 mg) and Tween 40 (400 mg). The chloroform in the mixture was evaporated, followed by addition of oxygenated ultra-pure water (100 ml) to prepare the β-carotene/linoleic acid emulsion. The emulsion was mixed well and the initial absorbance of the emulsion was measured at 470 and 700 nm.

Aliquots of the emulsion (3 ml) were added to the extracts (10–100 μl), and were then incubated at 50 °C for 60 min. The absorbance measurements were taken and antioxidant activity was calculated according to the formula reported by Lim and Quah (2007). In this assay, quercetin was used as a positive control.

2.12. Statistical analysis

All measurements were carried out in triplicate. Statistical analyses were performed using a one-way analysis of variance ANOVA, and the significance of the difference between means was determined by Duncan's multiple range test. Differences at $P < 0.05$ were considered statistically significant. The results were presented as mean values ± SD (standard deviations).

3. Results and discussion

3.1. Extraction solvent selection and extraction efficiency (EE) determination

Suitable extraction solvent was selected in order to achieve higher extraction yield. Studies reported that methanol showed better recoveries (Yao et al., 2004) and it is specifically effective in extracting polyphenols (Pinelo, Rubilar, Sineiro, & Núñez, 2004).

The TPC of the extracts in 75% and 100% methanol were investigated, and Table 1 shows TPC yields were comparable in all extracts, except the *C. surattensis* leaf. Consequently, 75% methanol was selected for subsequent antioxidant activities assays.

The average extraction efficiency (EE) using 75% methanol of all samples was determined by multiple extractions (three times). Table 1 shows that high EE was observed in the first extraction of all samples, ranging from 83% to 90%. This shows that most antioxidants were recovered during the first hour of extraction.

3.2. Total phenolic content (TPC), total anthocyanin content (TAC) and antioxidant activities

Several *in vitro* chemical assays were employed in this study, and a comparative evaluation of the phenolic and flavonoid compounds was performed. The Folin–Ciocalteu (F–C) assay has been

Table 1
Comparison of TPC of flowers (F) and leaves (L) of *B. kockiana*, *C. pulcherrima* and *C. surattensis* extracted in 100% methanol and 75% aqueous methanol and the extraction efficiencies (EE) in 75% aqueous methanol.

Plant	Part	TPC (mg GAE/100 g)		Average EE (%) based on first extraction
		100% methanol	75% aqueous methanol	
B. kockiana	F	7150 ± 691[a]	7540 ± 802[a]	83.9 ± 1.4
	L	4140 ± 165[a]	4440 ± 232[a]	87.6 ± 1.7
C. pulcherrima	F	2340 ± 182[a]	2380 ± 220[a]	83.3 ± 1.5
	L	4100 ± 373[a]	4830 ± 406[a]	89.8 ± 1.1
C. surattensis	F	2580 ± 374[a]	2780 ± 354[a]	86.0 ± 1.0
	L	2340 ± 75[a]	3065 ± 262[b]	83.3 ± 2.4

TPC is based on triplicates from a single batch. Results are expressed as means ± SD ($n = 3$). For each row, values followed by the same letter (a–b) are statistically insignificant ($P < 0.05$) as determined by ANOVA. The average EE is based on triplicates from a single batch extracted with 75% aqueous methanol, and only EE from first extraction were reported. Results are expressed as means ± SD ($n = 3$).

widely used to measure polyphenol contents, with the basic mechanism of electron transfer and reducing ability (Prior, Wu, & Schaich, 2005). Using this quantitative assay, we found that the TPC of flowers could be ranked as follows: *B. kockiana* > *C. surattensis* > *C. pulcherrima* (Table 2). On the other hand, the *C. pulcherrima* leaf has the highest TPC (5030 ± 602 mg GAE/100 g), followed by *B. kockiana* (4220 ± 104 mg GAE/100 g) and *C. surattensis* (3330 ± 309 mg GAE/100 g).

Antioxidant activities of the selected plants were also investigated using DPPH radical scavenging and FRP assays. DPPH is a compound consists of a nitrogen free radical which is easily quenched by a free radical scavenger. Within the presence of a proton radical scavenger or hydrogen donating antioxidants, DPPH radicals will be transformed into a non-radical form (DPPH-H) and the reduction of DPPH radicals can be measured at 517 nm (Prior et al., 2005). FRP is used to study the ability of the antioxidants in reducing iron (III) to iron (II) in a redox-linked reaction, involving a single electron transfer mechanism (Chew et al., 2008). Antioxidant activity determined using DPPH radical scavenging assay is expressed in terms of AEAC and IC_{50}, the concentration of extract needed to achieve 50% scavenging of DPPH radical under experimental condition. In this study, it was found that the AEAC and FRP of the plants were ranked similarly as TPC (Table 2), and positive correlations were found between TPC and AEAC ($R^2 = 0.9750$), and with FRP ($R^2 = 0.9567$), but a negative association was demonstrated between TPC and IC_{50} ($R^2 = 0.8362$). The correlations of these three antioxidant assessments were also found in studies on other plants, i.e. *Portulaca oleracea* (Lim & Quah, 2007), *Phyllanthus amarus* (Lim & Murtijaya, 2007), and several tropical seaweeds (Chew et al., 2008).

Statistical analysis performed showed that TPC, AEAC, IC_{50} and FRP of flowers and leaves from the same species were statistically different from each other. The only exception was *C. surattensis*, where the values of the flower and leaf were comparable (Table 2).

Anthocyanins (ACYs) were detected in the flowers of *B. kockiana* (32.5 ± 4.1 mg cy-3-glu/100 g) and *C. pulcherrima* (40.8 ± 4.4 mg cy-3-glu/100 g), but absent in *C. surattensis* and all the leaf extracts. ACY is a heterocyclic flavonoid, functioning as the colouring agent that gives the flowers a colouring from red through purple to blue (Narayan, Akhilender Naidu, Ravishankar, Srinivas, & Venkataraman, 1999). The colour pigment in *C. surattensis* flower is lutein, a carotenoid compound (Wang, 2008), which can also function as antioxidant.

A differential distribution of antioxidants was seen in *B. kockiana* and *C. pulcherrima*, while antioxidants in *C. surattensis* were uniformly distributed in the leaves and flowers. Siddhuraju, Mohan, and Becker (2002) reported that *Cassia fistula* leaves have higher phenolic content, reducing power and free radical inhibition activity than flowers due to the existence of several groups of polyphenol compounds such as anthraquinones, xanthones, proanthocyanidins and flavonol which could be contributing to the antioxidant activity measured. Close and McArthur (2002) stated that excessive light energy absorbed during photosynthesis would lead to the formation of triplet chlorophyll, singlet oxygen and hydroxyl radicals which are fatal to the leaf tissues as well as to the entire plant. Leaves would provide an oxidative protection so that the highly reactive chemical species and harmful by-products formed when excess light energy is received during photosynthesis could be quenched and removed effectively and efficiently. Besides, low antioxidant activity in flowers could be due to the existence of prooxidant substances that would exhibit suppressing effects in antioxidant capacity (Siddhuraju et al., 2002).

Another possible explanation is the different life span of the plant parts. Leaf is the primary photosynthetic organ which has a longer life span than the flower, the reproductive organ. Therefore, leaves are subjected to a higher level of daily and cumulative oxidative pressure, especially stress from the photosynthesis process than shorter-lived flowers. The production of higher antioxidant levels and capacity would prolong the viability of leaves.

However, the antioxidant activity of leaves may not always be higher than flowers. In our study, the *B. kockiana* flower was found to have a statistically significant higher phenolic content, as well as showing a stronger free radical scavenging activity and better FRP than the leaves. Significant higher antioxidant activity in flowers than leaves was also found in recent studies on British medicinal plants reported by Mantle, Eddeb, and Pickering (2000), and *Castanea sativa* (chestnut) by Barreira, Ferreira, Oliveira, and Pereira (2008). Barreira et al. (2008) explained that higher antioxidant activities exerted by the flowers is related to the types of antioxidant compounds in flowers (i.e. reductones) which are able to react with free radicals, stabilise and inhibit free radical chain reactions. Another possible explanation could be the role of ACYs as potential antioxidants. ACYs are usually synthesised in coloured flowers as visual characteristics to attract potential pollinators, but they also shield the plant tissues from oxidative damage at the same time.

3.3. Total flavonoid content (TFC) and polyphenols identified: In relation to TPC

The TFC of samples was measured according to the procedure of Chang et al. (2002). This assay is able to detect the existence of two types of flavonoids: flavones and flavonols. Results in Table 3 show that all extracts showed a positive reaction towards the TFC assay, except for the *B. kockiana* flower. The TFC of the leaves was ranked as follows: *C. pulcherrima* > *B. kockiana* > *C. surattensis* (Table 3). Flavonoid content was also found in flower extracts of *C. pulcherrima* and *C. surattensis*. A significant difference was observed between flowers and leaves of the same species. In addition, a variation ($P > 0.05$) was also seen when flowers and leaves from different species were compared.

To obtain the relationship between TFC and rutin content, TPC was also expressed in terms of mg rutin equivalents (RE) per 100 g fresh material, with the calibration equation for rutin, $y = 0.055x + 0.0252$ ($R^2 = 0.9903$), where x is the rutin concentration in mg/L, and y is the absorbance at 765 nm. It was observed that the TFC in relation to TPC_{Rutin} ranged from 1.87% (in *C. suratt-*

Table 2
Total phenolic content (TPC) and various antioxidant activities of flowers (F) and leaves (L) of *B. kockiana*, *C. pulcherrima* and *C. surattensis*.

Plant	Part	TPC (mg GAE/100 g)	IC_{50} (mg/ml)	AEAC (mg AA/100 g)	FRP (mg GAE/g)	TAC (mg cy-3-glu/100 g)
B. kockiana	F	8280 ± 498^a	0.027 ± 0.005^a	14,600 ± 2360^a	72.4 ± 8.7^a	32.5 ± 4.1
	L	4220 ± 104^b	0.061 ± 0.010^b	6410 ± 985^b	24.2 ± 2.8^b	ND
C. pulcherrima	F	2630 ± 338^a	0.118 ± 0.024^a	3350 ± 618^a	14.0 ± 2.7^a	40.8 ± 4.4
	L	5030 ± 602^b	0.050 ± 0.005^b	7690 ± 735^b	32.9 ± 2.6^b	ND
C. surattensis	F	3820 ± 342^a	0.096 ± 0.016^a	4080 ± 728^a	14.3 ± 2.5^a	ND
	L	3330 ± 309^a	0.094 ± 0.011^a	4130 ± 463^a	16.7 ± 2.5^a	ND

Results are expressed as means ± SD ($n = 3$). For each column, values followed by the same letter (a–b) are statistically insignificant ($P > 0.05$) as determined using ANOVA, and this does not apply for different plants. 'ND' represents 'not detected'.

Table 3
Total flavonoid content (TFC), rutin and chlorogenic acid composition of flowers (F) and leaves (L) of *B. kockiana*, *C. pulcherrima* and *C. surattensis*.

Plant	Part	TPC_{Rutin} (mg RE/ 100 g)	TFC (mg RE/100 g) (TFC/TPC_{Rutin})	Rutin content (mg/100 g) ($Rutin/TPC_{Rutin}$)	Chlorogenic acid content (mg/100 g)
B. kockiana	F	1540 ± 102^a	ND	ND	ND
	L	788 ± 20^b	91.2 ± 10.2^b (11.6%)	144 ± 7 (18.3%)	ND
C. pulcherrima	F	468 ± 71^a	46.2 ± 6.3^a (9.88%)	32.0 ± 1.3 (6.84%)	ND
	L	953 ± 121^b	161 ± 18^b (16.9%)	669 ± 26 (70.2%)	ND
C. surattensis	F	710 ± 69^a	13.3 ± 2.4^a (1.87%)	19.4 ± 0.7 (2.73%)	ND
	L	641 ± 63^a	29.6 ± 4.7^b (4.62%)	95.7 ± 13.5 (14.9%)	9.13 ± 0.44

Results are expressed as means ± SD ($n = 3$). For each column, values followed by the same letter (a–b) are statistically insignificant ($P > 0.05$) as determined by ANOVA, and this does not apply for different plants. 'ND' represents 'not detected'.

ensis flower) to 16.9% (in *C. pulcherrima* leaf) (Table 3), reflecting that low to moderate amounts of flavonoids were contributing to the polyphenol content in these plants.

For the *B. kockiana* flower, a negative response in the TFC assay reflected the absence or undetectable amount of flavones and flavonols. This implies that a high TPC and strong antioxidant activity measured in various chemical assays could be attributed by other types of polyphenols synthesised in the flower which are yet to be discovered.

As the TFC assay is incapable of identifying the individual flavonoids, HPLC analysis was carried out for specific detection and identification. The analysis found that rutin was present in most extracts, ranging from 19.4 ± 0.7 mg rutin/100 g plant material (in *C. surattensis* flower) to 669 ± 26 mg rutin/100 g plant material (in *C. pulcherrima* leaf), but it was absent in the *B. kockiana* flower (Table 3). The percentage of rutin content quantified in relation to TPC_{Rutin}, ranged from 2.73% (in *C. surattensis* flower) to 70.2% (in *C. pulcherrima* leaf), reflecting the unequal distribution of rutin, in relation to the polyphenol content in flowers and leaves of the same and different species.

In contrast, only minute amounts of chlorogenic acid were detected in the *C. surattensis* leaf (9.13 ± 0.44 mg/100 g leaves).

Duke, Bogenschutz-Godwin, duCellier, and Duke (2002) reported that ellagic acid, gallic acid, myricetin, quercetin and rutin were present in *C. pulcherrima*. Contrary to our findings where only rutin was found, the other two phenols (ellagic acid and gallic acid) and flavonoids (myricetin and quercetin) were absent. However, details of the study, i.e. location, parts and extraction method were not stated by the authors. This difference may be explained by the fact that different parts of the plant were studied. Apart from that, many studies have shown that various abiotic factors, i.e. plant origin, growing season, environmental stress, and growing location could influence the antioxidant content (Howard, Pandjaitan, Morelock, & Gil, 2002; Kirakosyan et al., 2004; Oleszek et al., 2002; Yu & Zhou, 2004). Consequently, one or a combination of several abiotic factors would possibly enhance or suppress phenylpropanoid synthesis pathways, resulting in deviation in polyphenol content.

3.4. Ferrous ion chelating activity

Presence of transition metal ions in a biological system could catalyse the Haber–Weiss and Fenton-type reactions, resulting in generation of hydroxyl radicals ($OH^{\cdot}$). However, these transition metal ions could form chelates with the antioxidants, which result in the suppression of $OH^{\cdot}$ generation, and inhibit peroxidation processes of biological molecules. Ferrous ion was employed as the transition metal ion in this FIC assay.

Analysis of ferrous ion chelating activity of these extracts shows a dose dependent FIC activity. As shown in Fig. 1, at the highest extract concentration (approximate 7 mg/ml), the *C. pulcherrima* leaf exhibited the highest FIC activity (⩾80%). The *C. surattensis* leaf displayed medium chelating activity (19–56%) and the *B. kockiana* leaf and all flower extracts showed the lowest activity (⩽40%). FIC values of flowers were comparable at 7 mg/ml, and no correlation

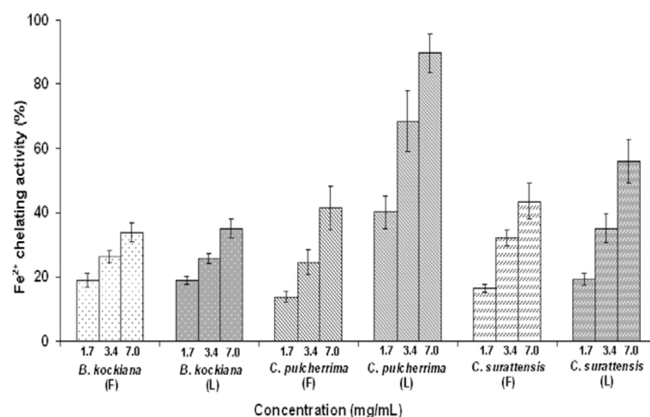

Fig. 1. Ferrous ion chelating activity of flowers (F) and leaves (L) of *B. kockiana*, *C. pulcherrima* and *C. surattensis*. Data are expressed as means ± SD ($n = 3$).

was seen between FIC and TPC (as well as with other antioxidant activities). It is interesting to note that although, the *B. kockiana* flower showed the highest values in TPC, AEAC and FRP, it exhibited the lowest ferrous ion chelating property. FIC activity is similar in the *B. kockiana* flower and leaf, in spite of significant variations in TPC, antioxidant activity and TFC. Similarly, little variations in chelating ability was seen in the *C. surattensis* flower and leaf. Studies showed that the metal ion binding efficiency is strongly related to the spatial conformation of the compounds present, as well as the position and number of electron-donating ligating groups (Khokhar & Owusu Apenten, 2003; Mira et al., 2002).

3.5. β-Carotene bleaching (BCB) assay

Assessment of antioxidant activity using the BCB assay is based on *in vitro* bleaching of β-carotene, caused by radicals released upon the oxidation of linoleic acid in the emulsion (Koleva, van Beek, Linssen, de Groot, & Evstatieva, 2002). The bleaching of β-carotene could be inhibited by antioxidants, which are capable of reducing the rate of chain reaction initiated during lipid peroxidation and transforming the reactive end product to a more stable form. Koleva et al. (2002) commented that the lipid peroxidation activity in the β-carotene-emulsion systems was inhibited by apolar antioxidants, where the hydrophobicity characteristics of these antioxidants are able to exhibit greater inhibition activity, shielding the emulsion by concentrating at the lipid:air surface. The effectiveness of antioxidants in suppressing the action of radicals towards β-carotene was evaluated by monitoring the colour reduction spectrophotometrically.

As seen in Fig. 2, the inhibition activity of extracts correlated positively with the mass of sample. Both flowers and leaves exhibited strong antioxidative properties in emulsion. The *C. pulcherrima* leaf displayed the greatest inhibition effect (98.6 ± 2.6%), and the *C. surattensis* flower demonstrated the lowest activity (89.5 ± 4.0%) at

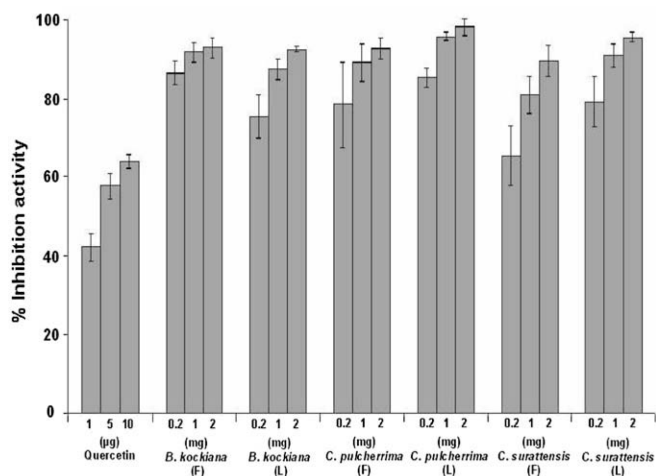

Fig. 2. Antioxidant activity of flowers (F) and leaves (L) of *B. kockiana*, *C. pulcherrima* and *C. surattensis* as determined by beta-carotene bleaching (BCB) assay. Data are expressed as means ± SD ($n = 3$).

2 mg plant material. Overall, all extracts displayed great potential of quenching linoleate free radicals (generated from linoleic acid peroxidation) and shielding the carotenoid from bleaching.

Besides apolar antioxidants, ACYs in plants could also exhibit a lipid peroxidation inhibition effect in this experimental system. Narayan et al. (1999) reported that ACYs are capable of inhibiting both enzymatic and non-enzymatic lipid peroxidation by a non-competitive mechanism and in a concentration dependent manner. They suggested that ACYs could bind with fatty radicals and inhibit the radical chain reaction, and consequently the lipid peroxidation process is terminated. In addition, flavonoids present in the plants could also scavenge O_2^-, and donate an H-atom to peroxyl radicals which would either inhibit or stop the radical chain reactions in the lipid peroxidation processes (Narayan et al., 1999).

It was observed that samples which showed higher chelating activity (viz. *C. pulcherrima* and *C. surattensis* leaves) displayed a better inhibition effect in the BCB assay. On the other hand, samples which showed similar metal ion chelating activity (viz. *B. kockiana*, *C. pulcherrima* and *C. surattensis* flowers and *B. kockiana* leaf) also demonstrated comparable lipid peroxidation inhibition ability.

4. Conclusion

This study showed that the selected medicinal plants from Leguminosae family are high in polyphenol contents. Rutin was found to be abundant in *C. pulcherrima* leaves. A positive correlation was found between TPC and various antioxidant activities assessed in *in vitro* systems, indicating that polyphenols could be the major contributors to free radical scavenging and reducing ability of the extracts. A high lipid peroxidation inhibition activity of extracts suggested that their antioxidants could be utilised as alternative food ingredients and preservatives.

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