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Quantification of rutin, quercitrin and quercetin in *Cosmos caudatus* Kunth by reverse phase high performance liquid chromatography

M.M.A. Sharifuldin[1], Z. Ismail[1], A.F.A. Aisha[1], E.K. Seow[2] and H.K. Beh[3*]

[1]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia; [2]School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia; [3]Faculty of Agro Based Industry, Universiti Malaysia Kelantan, Kelantan, Malaysia; hooikheng@umk.edu.my

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RESEARCH ARTICLE

Abstract

Cosmos caudatus is a traditional medicinal plant from the family *Compositae*. The fresh leaves are consumed as appetizer and for health benefits such as antioxidant activity, to improve blood circulation and to promote the formation of healthy bones. Our study aims to develop a reverse phase high performance liquid chromatography method for quantification of rutin, quercitrin and quercetin in *C. caudatus* extracts. The method was validated for selectivity, linearity, precision, accuracy and limits of detection and quantification. Selectivity was confirmed based on retention time and ultraviolet-visible spectra; linearity was in the range 0.5-500 µg/ml at R^2=1.0; the intraday and interday precision was determined as the relative standard deviation of peak area and retention time. Percentage recovery, limits of detection and the limits of quantification of rutin, quercitrin and quercetin were also determined. The reported method was applied for the quantification of the 3 marker compounds in 15 extracts of *C. caudatus*. Results showed quercitrin as the major constituent in *C. caudatus;* hence it may be used as a reference compound for standardisation of this medicinal herb. The method can be considered as analytical tool for quality control, stability studies, pharmacokinetics, and standardisation purposes.

Keywords: medicinal plant, extracts, method validation, marker compounds, quality control assurance.

1. Introduction

Cosmos caudatus Kunth is a traditional vegetable in Malaysia that usually consumed as '*ulam*'. Previous studies showed that different varieties of Malaysian vegetables are rich in carbohydrates, proteins, minerals and vitamins (Abas *et al.*, 2006). Antioxidant activity of *C. caudatus* was also reported due to the high concentration of flavonoids such as quercetin and quercitrin (Abas *et al.*, 2003; Andarwulan *et al.*, 2010; Huda-Faujan *et al.*, 2007, 2009; Mustafa *et al.*, 2010; Wong *et al.*, 2006).

Previous phytochemical studies on *C. caudatus* led to the isolation of compounds such as quercetin-3-O-β-arabinofuranoside, quercetin-3-O-α-rhamnoside, quercetin-3-O-β-glucoside, and quercetin, which showed strong antioxidant activity (Abas, 2005). Mediani *et al.* (2012) identified six compounds including quercetin-3-O-rhamnoside, quercetin-3-O-glucoside, rutin, quercetin-3-O-arabinofuranoside, quercetin-3-O-galactoside and chlorogenic acid in *C. caudatus*. Their study showed significant variance of the compounds' concentrations at different age of herbs (8, 10 and 12 weeks-old).

Few analytical methods have been reported for detection and quantification of flavonoids, however the available methods are either time consuming or have been developed for analysis of some bioactive compounds only (Boligon *et al.*, 2012; Dubber and Kanfer, 2004; Li *et al.*, 2007). Chromatographic technique was regarded as a useful method to control the quality of the herbal medicines and their derivatives. The chromatographic methods include high performance liquid chromatography (HPLC), gas chromatography and thin-layer chromatography. HPLC analysis has been regarded as the first choice due to precision, sensitivity and reproducibility (Chen *et al.*, 2008).

Due to the increased demand of this herb, a fast and reliable analytical method is needed for the quantification of its bioactive compounds. HPLC analysis method could be useful for routine standardisation and quality control issues, which are critical to the herbal producing industry. For example quality control during the process of production, checking the genuine origin and proper storage condition of the herbs, as well as detection of adulterants. Therefore, the present study was conducted in order to develop and validate a new reverse phase high performance liquid chromatography method (RP-HPLC) for the quantification of three major flavonoid compounds (rutin, quercitrin and quercetin) in various *C. caudatus* extracts.

2. Materials and methods

Preparation of raw material

C. caudatus commercial samples were provided by a specialised company in herbal products (Herbagus Sdn Bhd.), from 3 different locations in Malaysia: Penang Island (located on the northwest coast of Peninsular Malaysia by the Strait of Malacca), Selangor (located in the centre of Peninsular Malaysia and the Strait of Malacca to the west), and Johor (the southernmost state of the Peninsular Malaysia). The collected plant material was washed with tap water, oven-dried at 50 ˚C for 2-3 days, and grinded to the powder form using electric grinder.

Chemicals and reagents

Rutin and quercetin reference compounds were obtained from Sigma-Aldrich (Kuala Lumpur, Malaysia) and quercitrin was purchased from HWI Analytik GmbH (Ruelzheim, Germany). The solvents were of HPLC or analytical grades, and were acquired from Merck Sdn. Bhd. (Petaling Jaya, Selangor, Malaysia). The reverse phase Acclaim Polar Advantage II, (150 × 4.6 mm × 5.0 µm) was purchased from Dionex (Sunnyvale, CA, USA).

Extraction

Five extracts of *C. caudatus* were prepared in 96%, 75%, 50% and, 25% ethanol and water. Extraction was carried out using soxhlet extractor for the ethanolic extracts and reflux for the water extract, the solvent:solid ratio was set at 16:1 (v/w) and extraction time was 48 h. Extracts were concentrated at 60 °C using a rotary evaporator and further dried in freeze-drier.

Instrumentation and high performance liquid chromatography conditions

A Dionex-Ultimate® 3000 Rapid Separation LC system (Dionex, Seri Kembangan, Selangor, Malaysia) was used. The instrument is equipped with auto sampler, quaternary pump, degasser, column oven, and a DAD-3000RS diode array detector (DAD) detector. The chromatographic analysis was performed on a reverse phase Acclaim Polar Advantage II column (150 × 4.6 mm × 5.0 µm; Dionex). The column temperature was set at 40 °C, the mobile phase was consisting of A (0.3% formic acid in water) and B (acetonitrile), the elution program was gradient for 20 min, the flow rate was maintained at 1 ml/min, and the injection volume was 10 µl. The spectral data from the DAD was collected at 254 nm and data acquisition was performed by Chromeleon software version 6.8 (Dionex).

Preparation of the standard mixture

A stock solution consisting of a mixture of rutin, quercitrin and quercetin reference compounds was prepared at 2 mg/ml in HPLC grade methanol. The solution was further diluted to obtain 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.98, 0.49 µg/ml. The sample extracts were prepared at 10 mg/ml in the same solvent, and were further diluted to obtain 1 mg/ml. Stock solutions were filtered through 0.45 µm syringe filters.

Method validation

The proposed method was validated according to the ICH guidelines (ICH, 1997). The following validation characteristics were evaluated: selectivity, linearity, precision, accuracy and the limits of detection and quantification (LOD and LOQ).

Linearity

Linearity was determined by injecting 10 µl of the standard mixture in a concentration range of 0.5-500 µg/ml. The calibration curves were obtained for each by plotting the peak area versus the concentration. Linearity (R^2) was determined by regression analysis of the calibration graphs.

Selectivity

The selectivity was determined by comparing the retention time and the ultraviolet-visible spectra of the target compounds obtained in the sample extracts with their reference counterparts.

Precision

Precision was determined as the percentage relative standard deviation (%RSD) of the peak area and retention time. The standard mixture was analysed at 7 concentration points in the range 3.9-250 µg/ml, and the intraday and interday precision was determined (n=6).

Accuracy

Accuracy was determined as a percentage recovery of the 3 target compounds (rutin, quercitrin and quercetin) at 12.5, 25 and 50 µg/ml added to the ethanolic extract at 10 µg/ml. The peak area corresponding to the compounds in the ethanolic extract (B), the individual reference compounds (C) and their combinations (A) was recorded. The percentage recovery was then calculated using the following formula (Aisha *et al.*, 2012):

$$\%Recovery = ((A - B) / C) \times 100$$

The results are presented average ± standard deviation (SD) (n=3).

Limits of detection and quantification

The LOD and LOQ were calculated through the slope and standard deviation method as described previously (ICH, 1997):

$$LOD = (3.3 \times \delta) / S \text{ and}$$

$$LOQ = (10 \times \delta) / S$$

Where:
δ: is the standard deviation of the Y intercept of the linear regression equations.
S: is the slope of the linear regression equations.

Determination of rutin, quercitrin and quercetin concentration in *Cosmos caudatus* extracts

10 µl of *C. caudatus* extracts were injected at 1000 µg/ml, and the peak area corresponding to rutin, quercitrin and quercetin was recorded. The linear regression equations of the standard calibration curves were applied to in order calculate the concentration of the marker compounds in the samples, and the results are presented as a %w/w using the formula:

$$\%w/w = (\text{the found concentration} / 1000\ \mu g/ml) \times 10\ (n=3)$$

3. Results and discussions

WHO encouraged governments to effectively utilise local knowledge of herbal medicines for disease prevention and health promotion. Herbal medicines, however, suffer from a range of shortcomings. These include insufficient and unacceptable evidences of safety, efficacy and standardisation practices (Mosihuzzaman and Choudhary, 2008). The number of reports of patients experiencing negative health consequences caused by the use of herbal medicines has increased. One of the major causes of reported adverse events is directly linked to the poor quality of herbal medicines (Mendonca-Filho, 2006). Hence, there is an urgent need of effective standardisation method and referential information for herbal materials. The present study developed and validated a fast and new RP-HPLC for the quantification of three major flavonoid compounds (rutin, quercitrin and quercetin) in various *C. caudatus* extracts. The validated method enables an efficient and productive use of the process and instrumental variables.

Selectivity

The selectivity of the method was determined firstly by comparing the retention time of rutin, quercitrin and quercetin obtained in the sample extracts with those of the reference compounds; the retention time of the reference compounds was 7.78±0.01, 9.87±0.01, and 15.59±0.02 min (Supplementary Figure S1), and that their counterparts in *C. caudatus* extracts was 7.75±0.01, 9.85±0.01, and 15.59±0.01 min, respectively.

Linearity

Linearity was presented in terms of regression coefficient (R^2) of reference compounds regression equations. R^2 was 1.0 in the 3 reference compounds which indicates good linearity of the proposed method.

Precision

Precision refers to the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. This is related to the random error of a measurement process (Cavalier, 2008). The first type is repeatability or intra-assay precision. Intra-assay precision data were obtained by repeatedly analysing, in one laboratory on 1 day, with a minimum of three aliquots homogeneous sample, each of which was independently prepared according to the method procedure. The second type is intermediate precision. These data were obtained by repeating the intra-assay experiment on a different day with newly prepared mobile phase and samples (Marin *et al.*, 2002). The precision analysis of this study is presented in terms of the %RSD of the retention time and peak area of rutin, quercitrin and quercetin reference compounds. The compounds were eluted at 7.78±0.01, 9.87±0.01 and 15.59±0.02 min, respectively. The %RSD was <0.2%. The %RSD of the peak area was calculated in the concentration range 0.19-100 µg/ml, and the average %RSD value was <0.3% (Table 1). These results indicate good reproducibility of retention time and peak area.

Table 1. Precision analysis and accuracy of the high performance liquid chromatography (HPLC) method.[1]

Compounds	Intraday data	Interday data	Average	%recovery		
				12.5 µg/µl	25 µg/µl	50 µg/µl
Rutin	0.17±0.06	0.17±0.13	0.17±0.00	94.9±1.8	95.9±3.0	91.6±3.1
Quercitrin	0.25±0.13	0.31±0.15	0.28±0.04	80.4±1.5	93.3±2.8	92.8±2.6
Quercetin	0.32±0.19	0.30±0.22	0.31±0.01	98.1±2.6	98.0±3.3	94.3±2.7

[1] The relative standard deviation of the peak area was calculated in the intraday and interday data, and the results are presented as average ± standard deviation (SD) (n=6). Accuracy of the HPLC method in the concentration range 12.5-50 µg/ml. Results are shown as average a percentage recovery of the reference compounds ± SD (n=3).

Accuracy and recovery

Accuracy is the closeness of agreement between the values, which is accepted either as a conventional true value or an accepted reference value and the value found in the study. It therefore refers to total measurement error (Cavalier, 2008). The accuracy is acceptable if the agreement of true and observed values is less than 15%. The accuracy of this method was evaluated, by the external standard method, as a percentage recovery of the reference compounds at 12.5, 25 and 50 µg/ml (Table 1).

Limit of detection and limit of quantification

Sensitivity of the method is evaluated by the LOD and LOQ. In this study, the linear regression equations of the reference compounds along with the LOD and LOQ values are presented in Table 2.

Concentration of rutin, quercitrin and quercetin in *Cosmos caudatus* extracts

To measure the quality assurance of the herbal material consisting of active phytochemicals is the critical parameter. They are number of factor that may cause changes in their chemical constituents which is agro-climatic factors, geographical variations, harvesting time, post-harvesting handling and storage of raw materials (Jadhav *et al.*, 2003). Fifteen extracts of *C. caudatus* from different locations and extracted with different solvents were analysed (Supplementary Figure S2).

The concentration of rutin, quercitrin and quercetin in the extracts was calculated by applying the linear regression equations of reference compounds, and the results are presented as average %w/w ± SD. The results showed that quercitrin is the main flavonoid component of the extracts, with lower concentration of rutin and quercetin. The highest concentration of quercitrin was obtained in the ethanol extract, whereas rutin and quercetin were obtained at higher concentration in the 50% ethanolic extract (Table 3).

In the present study, *C. caudatus* extracts were standardised versus rutin, quercitrin and quercetin. The compounds have been selected as standards in quality control of this herb because they are the major flavonoid components of this herb, which may explain its antioxidant activity. Leong and Shui (2001) reported antioxidant activity capacity of 27 selected fruits. The fruits comprised ciku (*Manilkara zapota*), strawberry (*Fragaria virginiana*), 'Flame seedless' grape (*Vitis vinifera*), guava (*Psidium guajava*), plum (*Prunus domestics*), star fruit (*Averrhoa carambola* L.), kiwi fruit (*Actinidia chinensis*), mango (*Mangifera indica* L.), lemon (*Citrus limon*), papaya var. solo (*Carica papaya* L.), mangosteen (*Garcinia mangostana*

Table 2. Summary of the calibration data of the reference compounds.[1]

Compounds	a	b	Limit of detection (µg/ml)	Limit of quantification (µg/ml)	R^2
Rutin	0.2898±0.0003	0.0185±0.010	0.0496±0.003	0.1503±0.009	1.0000
Quercitrin	0.2205±0.0001	0.0346±0.003	0.0777±0.001	0.2355±0.001	1.0000
Quercetin	0.5374±0.0003	0.4045±0.050	0.1581±0.008	0.4792±0.030	1.0000

[1] The regression equation is (y = ax + b), where (a) is the slope and (b) is the y intercept. The data are presented as average ± standard deviation (n=6).

Table 3. Rutin, quercitrin and quercetin content in *Cosmos caudatus* whole plant extracts.[1]

Extracts[2]	Rutin	Quercitrin	Quercetin
96% ethanol B	0.38±0.05	13.11±0.16	0.26±0.02
96% ethanol p	0.47±0.01	11.80±0.20	0.92±0.01
96% ethanol J	0.54±0.12	13.78±0.18	0.30±0.04
75% ethanol B	0.34±0.09	8.14±0.67	0.40±0.03
75% ethanol P	0.94±0.01	13.00±0.15	0.51±0.02
75% ethanol J	0.47±0.05	8.16±0.48	0.31±0.05
50% ethanol B	0.48±0.01	8.98±0.23	0.62±0.02
50% ethanol P	0.47±0.07	6.60±0.67	0.99±0.03
50% ethanol J	0.42±0.01	5.72±0.09	0.48±0.01
25% ethanol B	0.32±0.01	6.35±0.06	0.63±0.01
25% ethanol P	0.23±0.01	3.31±0.04	0.63±0.01
25% ethanol J	0.24±0.01	3.53±0.06	0.61±0.01
Water B	0.19±0.01	3.33±0.03	0.19±0.01
Water P	0.13±0.01	1.51±0.04	0.23±0.01
Water J	0.13±0.01	1.97±0.04	0.18±0.01

[1] Results are depicted as average %w/w ± SD (n=3).
[2] B, P, J refer to the different locations (Selangor, Penang, Johor respectively) from where the plants have been obtained.

L.), salak (*Salacca edulis*), avocado (*Persea Americana*), foot long papaya (*C. papaya* L.), pomelo (*Citrus grandis*), orange (*Citrus aurantium*), cempedak (*Artocarpus integer* Merr.), rambutan king (*Nephelium mutabile*), rambutan (*Nephelium lappaceum* L.), apple (*Malus pumila*), pineapple (*Ananas comosus* Merr.), tomato (*Lycopersicon esculentum*), banana (*Musa paridasiaca*), rockmelon (*Cucumis melo* var. cantalupensis), honeydew (*C. melo* var. inodorus), watermelon (*Citrullus vulgaris*) and coconut (*Cocos nucifera*). The results showed that the L-ascorbic acid equivalent antioxidant capacity (AEAC) values of all fruits were lower than 500 mg AEAC per 100 g fresh sample except ciku fruit (3,396 mg AEAC per 100 g fresh sample). In 2005, the same group of the researchers investigated antioxidant activity capacity of *C. caudatus*. They found that it showed an extremely high antioxidant capacity with an AEAC value of 2,500 mg/100 g fresh sample, which was close to that of ciku fruit. The extremely high antioxidant capacity of ulam raja may be mainly, or at least partly, responsible for its medicinal uses (Shui *et al.*, 2005).

The HPLC chromatograms of *C. caudatus* showed quercitrin is the major compound compared to the other two compounds and it shows variations in chemical constituents for plants collected from different geographical zones using different solvent extraction. These 3 compounds can be used to authenticate *C. caudatus* plant extract in standardisation process. The use of acetonitrile and 0.3% formic acid as the mobile phase resulted the short elution time (<16 min) with a good separation of more than 6 compounds including the three targeted peaks. This method also can be used to quantify other separated peaks, and it gives flexibility to quantify extracts that contain high concentration and low concentration of targeted compounds as we used a wide concentration range (0.2-500 µg/ml). The concentration range provided by this study is important. Norazlina *et al.* (2013) reported high dose of *C. caudatus* aqueous extracts (500-2,000 mg/kg) elevated alkaline phosphatase and alanine transaminase levels in male rats. The extracts may cause acute hepatotoxicity at high dose. Takami *et al.* (2008) reported high content of polyphenols such as flavonoids, may cause an increase in liver enzymes. Therefore there is an urgent need to develop a fast and reliable method to quantify these chemical constituents in *C. caudatus*. Overall, we report a new RP-HPLC method for the quantification of rutin, quercitrin and quercetin in *C. caudatus* extracts. The reported method was found to be rapid, selective, precise, accurate and with high sensitivity. This method provides several advantages over the previously published methods as it required less elution time and hence less solvent, and the detection of three reference flavonoid compounds at one time.

4. Conclusions

The developed method is an useful application and has the ability to analyse flavonoids compounds in *C. caudatus* in a single analytical run and detect the compounds at low concentrations. The proposed method is selective and possessed high accuracy and precision, in a linear study range. The method also provided adequate parameters for detection and quantification of rutin, quercitrin and quercetin in *C. caudatus*. We believed this method is an important contribution to the research on *C. caudatus,* and could be considered as an analytical tool for quality control assurance of *C. caudatus* herbal products.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/QAS2015.0839.

Figure S1. The high performance liquid chromatography chromatogram of the reference compounds rutin, quercitrin and quercetin.

Figure S2. High performance liquid chromatography chromatograms of *Cosmos caudatus* extracts at 254 nm.

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References

Abas, F., 2005. Phytochemical and biological activity studies of *Cosmos caudatus* and *Curcuma mangga* and the online characterization of bioactive fractions from *Melicope ptelefolia*. PhD thesis, University of Putra, Malaysia.

Abas, F., Lajis, N.H., Israf, D.A., Khozirah, S. and Umi Kalsom, Y., 2006. Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables. Food Chemistry 95: 566-573.

Abas, F., Shaari, K., Lajis, N.H., Israf, D.A. and Kalsom, Y.U., 2003. Antioxidative and radical scavenging properties of the constituents isolated from *Cosmos caudatus* Kunth. Natural Product Sciences 9(4): 245-248.

Aisha, A.F.A., Abu-Salah, K.M., Siddiqui, M.J., Ismail, Z. and Majid, A.M.S.A., 2012. Quantification of α-, β- and γ-mangostin in *Garcinia mangostana* fruit rind extracts by a reverse phase high performance liquid chromatography. Journal of Medicinal Plants Research 6: 4526-4534.

Andarwulan, N., Batari, R., Sandrasari, D.A., Bolling, B. and Wijaya, H., 2010. Flavonoid content and antioxidant activity of vegetables from Indonesia. Food Chemistry 121: 1231-1235.

Boligon, A.A., Agertt, V., Janovik, V., Cruz, R.C., Campos, M.M.A., Guillaume, D., Athayde, M.L. and Dos Santos, A.R.S., 2012. Antimycobacterial activity of the fractions and compounds from *Scutia buxifolia*. Brazilian Journal of Pharmacognosy 22: 45-52.

Cavalier, E., Rozet, E., Dubois, N., Charlier, C., Hubert, P., Chapelle, J.P., Krzesinski, J.M. and Delanaye, P., 2008. Performance of iohexol determination in serum and urine by HPLC: Validation, risk and uncertainty assessment. Clinica Chimica Acta 396: 80-85.

Chen, Y., Yan, Y., Xie, M.Y., Nie, S.P., Liu, W., Gong, X.F. and Wang, Y.X., 2008. Development of a chromatographic fingerprint for the chloroform extracts of *Ganoderma lucidum* by HPLC and LC-MS. Journal of Pharmaceutical and Biomedical Analysis 47: 469-477.

Dubber, M.-J. and Kanfer, I., 2004. High-performance liquid chromatographic determination of selected flavonols in *Ginkgo biloba* solid oral dosage forms. Journal of Pharmaceutical Sciences 7(3): 303-309.

Huda-Faujan, N., Noriham, A., Norakkiah, A.S. and Babji, A.S., 2007. Antioxidative activities of waters extracts of some malaysian herbs. ASEAN Food Journal 14: 61-68.

Huda-Faujan, N., Noriham, A., Norrakiah, A.S. and Babji, A.S., 2009. Antioxidant activity of plants methanolic extracts containing phenolic compounds. African Journal of Biotechnology 8(3): 484-489.

International Conference on Harmonisation (ICH), 1997. Guidance for industry, q2b: validation of analytical procedures: methodology. ICH, USA. Available at: http://tinyurl.com/jzraea3.

Jadhav, R.B., Patil, C.R., Bhpoe, S. and Murumkar, C.V., 2003. Herbal drug standardization and quality assurance of raw materials: a rational approach. Natural Product Radiance 2: 134-140.

Leong, L.P. and Shui, G., 2001. An investigation of antioxidant capacity of fruits in Singapore markets. Food Chemistry 76: 69-75.

Li, J., Jiang, B., Liu, X., Zhang, J., Chen, X. and Bi, K., 2007. Simultaneous determination of five bioactive flavonoids in *Hypericum japonicum* Thunb by High-performance Liquid Chromatography. Asian Journal of Traditional Medicine 2: 75-81.

Marin, A., Garcia, E., Garcia, A. and Barbas, C., 2002. Validation of a HPLC quantification of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations: capsules and sachets. Journal of Pharmaceutical and Biomedical Analysis 29: 701-714.

Mediani, A., Abas, F., Tan, C.P., Khatib, A. and Nordin, H.L., 2012. Influence of growth stage and season on the antioxidant constituents of *Cosmos caudatus.* Plant Foods for Human Nutrition 67: 344-350.

Mendonça-Filho, R.R., 2006. Bioactive phytocompounds: new approaches in the phytosciences. In: Ahmad, I., Aqil, F. and Owais, M. (eds.) Modern Phytomedicine. Wiley-VCH, Weinheim, Germany.

Mosihuzzaman, M. and Choudhary, M.I., 2008. Protocols on safety, efficacy, standardization, and documentation of herbal medicine. Pure and Applied Chemistry 80: 2195-2230.

Mustafa, R.A., Hamid, A.A., Mohamed, S. and Bakar, F.A., 2010. Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants. Journal of Food Science 75: 28-35.

Norazlina, M., Ehsan, S.Z., Adilah, N.K., Lee, C.P., Farhana, E., Derick, P., Ima-Nirwana, S., Nazrun, A.S. and Norliza, M., 2013. Acute toxicity study of *Cosmos caudatus* on biochemical parameters in male rats. Sains Malaysia 42(9): 1247-1251.

Shui, G., Leong, L.P. and Wong, S.P., 2005. Rapid screening and characterization of antioxidants of *Cosmos caudatus* using liquid chromatography coupled with mass spectrometry. Journal of Chromatography B 827: 127-138.

Takami, S., Imai, T., Hasumura, M., Cho, Y.M., Onose, J. and Hirose, M., 2008. Evaluation of toxicity of green tea catechins with 90-day dietary administration to F344 rats. Food and Chemical Toxicology 46: 2224-2229.

Wong, S.P., Leong, L.P. and William Koh, J.H., 2006. Antioxidant activities of aqueous extracts of selected plants. Food Chemistry 99: 775-783.