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ESTIMATION OF CENTELLOIDS AND TOTAL PHENOLIC CONTENT OF *CENTELLA ASIATICA* (L.) URB. USING COMPUTER SOFTWARE AND THEIR CORRELATION WITH ANTIOXIDANT ACTIVITY

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Abstract: *Centella asiatica* (L.) Urb. is a herbal plant with wound healing property and several biological activities. The aims of this work were to estimate the centelloids and total phenolic content of *Centella asiatica* (L.) Urb. using computer software and investigate the linear correlation between centelloids or total phenolic content and the antioxidant activity. The two-factor spherical composite experimental design was performed to estimate the content of centelloids and total phenolic compound. Extraction temperature and extraction time were varied five levels from 40 to 60 °C and 60 to 120 min, respectively. The increment of temperature and time provided a high content of centelloids. The higher content of total phenolic compound was achieved at the high temperature and medium extraction time. The estimation equation of both two groups of compound fitted with quadratic model. The estimation was reliable and stable. The optimal condition provided the highest centelloids content was 59 °C and 108.1 min. While 55.7 °C and 77.4 min gave the highest total phenolic content. The correlation study using statistical software between centelloids or total phenolic content and antioxidant activity was performed, centelloids content had a negative effect on antioxidant activity from 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. But, no correlation was found from ferric ion reducing antioxidant power (FRAP) assay. Total phenolic content did not correlate with antioxidant activity from DPPH radical scavenging assay as well as FRAP assay. These results are valuable for our future work as a standard condition for extraction of *Centella asiatica* (L.) Urb. to obtain the high content of active chemical constituent.

Keywords: centelloids content, total phenolic content, Centella, computer software, antioxidant activity

บทคัดย่อ: บัวบกเป็นพืชสมุนไพรที่มีฤทธิ์สมานแผลและฤทธิ์ทางชีวภาพหลายประการ การศึกษานี้มีวัตถุประสงค์เพื่อประมาณค่าปริมาณสารเซนเทลลอยด์และปริมาณสารฟีนอลิกรวมในบัวบกโดยใช้ซอฟต์แวร์คอมพิวเตอร์ และประเมินความสัมพันธ์เชิงเส้นตรงระหว่างปริมาณสารเซนเทลลอยด์หรือปริมาณสารฟีนอลิกรวมกับฤทธิ์ต้านอนุมูลอิสระ การศึกษานี้ใช้การออกแบบการทดลองแบบคอมโพสิตทรงกลมสองปัจจัยเพื่อประมาณค่าปริมาณสารเซนเทลลอยด์และปริมาณสารฟีนอลิกรวม โดยปรับเปลี่ยนปัจจัยด้านอุณหภูมิและระยะเวลา 5 ระดับ จาก 40 ถึง 60 องศาเซลเซียส และ 60 ถึง 120 นาที ตามลำดับ การเพิ่มอุณหภูมิและระยะเวลาการสกัด ส่งผลให้ปริมาณสารเซนเทลลอยด์เพิ่มสูงขึ้น ในขณะที่ปริมาณสารฟีนอ ลิกรวมจะพบมากที่อุณหภูมิสูงและระยะเวลาการสกัดปานกลาง สมการสำหรับการประมาณค่าของสารทั้งสองกลุ่มเป็นแบบโมเดลควอดราทิก การประมาณค่านี้มีความน่าเชื่อถือและมีความเสถียร สภาวะเหมาะสมที่สุดของการสกัดเพื่อให้ได้ปริมาณสารเซนเทลลอยด์สูงสุด คือ อุณหภูมิ 59 องศาเซลเซียส และระยะเวลา 108.1 นาที ในขณะที่อุณหภูมิ 55.7 องศาเซลเซียส และระยะเวลา 77.4 นาที ทำให้ได้ปริมาณสารฟีนอลิกรวมสูงสุด การศึกษาความสัมพันธ์ด้วยซอฟแวร์ทางสถิติระหว่างปริมาณสารเซนเทลลอยด์หรือปริมาณสารฟีนอลิกรวมกับฤทธิ์ต้านอนุมูลอิสระ พบว่า ปริมาณสารเซนเทลลอยด์ทำให้ฤทธิ์ต้านอนุมูลอิสระลดลงจากการทดสอบด้วยวิธีการทำลายอนุมูลอิสระดีพีพีเอช แต่ไม่พบความสัมพันธ์จากการทดสอบด้วยวิธีวิเคราะห์ความสามารถในการรีดิวซ์เฟอร์ริกของสารต้านอนุมูลอิสระ ปริมาณสารฟีนอลิกรวมไม่มีความสัมพันธ์กับฤทธิ์ต้านอนุมูลอิสระที่ได้จากการทดสอบด้วยวิธีการทำลายอนุมูลอิสระดีพีพีเอชและวิธีวิเคราะห์ความสามารถในการรีดิวซ์เฟอร์ริกของสารต้านอนุมูล อิสระ การศึกษานี้มีความสำคัญอย่างมากซึ่งจะถือเป็นสภาวะมาตรฐานสำหรับการสกัดบัวบกในการศึกษาต่อไปเพื่อให้ได้ปริมาณสารสำคัญในปริมาณสูง

คำสำคัญ: ปริมาณสารเซนเทลลอยด์ ปริมาณสารฟีนอลิกรวม บัวบก ซอฟต์แวร์คอมพิวเตอร์ ฤทธิ์ต้านอนุมูลอิสระ

INTRODUCTION

Centella asiatica (L.) Urb. (Apiaceae) is an herbal medicinal plant which listed in Thailand National Essential Drug List with wound healing property. Nowadays, it is used in dermatology for treatment of several symptoms i.e. wound, psoriasis, scleroderma, bacterial infection, and antioxidation (Bylka *et al.*, 2014). Furthermore, it also uses as an adaptogen, central nervous system relaxant, peripheral vasodilator, sedative, antibiotic, detoxifier, blood-purifier, laxative, diuretic, emmenagogue (Khare, 2007). It contains numerous chemical compounds, however, pentacyclic triterpenoids called centelloids (madecassoside, asiaticoside, madecassic acid, and asiatic acid) is an important compound that has wound healing activity (Bylka *et al.*, 2014). *C. asiatica* also contains a phenolic compound which is a well-known dietary phytochemical with antioxidant activity. It usually found in fruits, vegetables, and grains. The scientific evidence suggests that it have protective effects against several degenerative diseases (Giada, 2013; Pandey and Rizvi, 2009).

Recently, the modern experimental design has a superior advantage than traditional one-factor design i.e. less time, material, and financial consuming, interaction effect can be identified, and surface response can be characterized (Gibson, 2016). Design-Expert® is a user-friendly software that effective in the estimation of extraction of active chemical constituent from plants e.g. *Pueraria thomsonii* (Liu *et al.*, 2011), *Gentiana rigescens* (Pan *et al.*, 2015), *Sargassum thunbergii* (Yuan *et al.*, 2015), *Melaleuca bracteata* (Hou *et al.*, 2016), *Ixora siamensis* (Mat Nor and Arof, 2016), etc.

The objectives of this work were to estimate the centelloids and total phenolic content of *C. asiatica* using computer software and investigate the linear correlation between centelloids or total phenolic content and the antioxidant activity obtain from two assays; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric ion reducing antioxidant power (FRAP) assay. The authors expected that the results might be used as a standard procedure for extraction of an active chemical constituent to obtain the highest content of each compound.

MATERIALS AND METHODS

Materials

Asiaticoside, madecassic acid, and asiatic acid were purchased from Chengdu Biopurify Phytochemicals Ltd., China. Madecassoside, gallic acid monohydrate, Folin-Ciocalteu's phenol reagent, DPPH, and 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Sigma-Aldrich Inc., USA. Ferric (III) chloride hexahydrate ($FeCl_3 \bullet 6H_2O$) was purchased from Merck, Germany. Acetonitrile (HPLC grade) was purchased from Honeywell-Burdick & Jackson, USA. Orthophosphoric acid was purchased from Carlo-Erba, France. Ultrapure water was produced by the Puris-Expe UP system, Korea. The other chemicals were analytical grade.

Plant sample

Arial part of *C. asiatica* was harvested from Sai Noi District, Nonthaburi Province, Thailand in March 2017. It was authenticated by Chair Prof. Dr. Nijsiri Ruangrungsi, Faculty of Pharmacy, Rangsit University to confirm the correct plant species. Plant sample was deposited at the Herbal Medicinal Products Research and Development Center, Faculty of Pharmacy, Rangsit University (voucher specimen no. CM-CA001-1-03-2017).

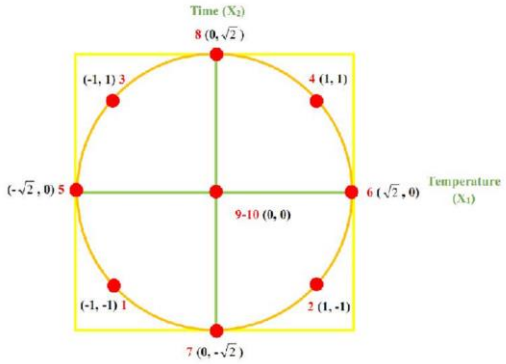
Plant sample preparation and extraction

Plant sample was washed to remove foreign matter, air dried, and then dried in hot air oven (JSOF-100, JS Research Inc., Korea) at 50 °C for 24 h. It was pulverized, passed through a 60-mesh sieve and kept until use in ambient condition with light and excess humidity protection.

Plant powder (20 g) was added to 250-mL Erlenmeyer flask. Then, 100 mL of 95% ethanol was filled. It was extracted using a water bath (WNB-14, Memmert, Germany) with specific temperature and time as showed in Figure 1. The filtrate was separated from the marc by vacuum filtration through Whatman filter paper no.1. The marc was extracted again for additional two times. The three parts of filtrate were pooled and evaporated using rotary evaporator (Büchi Labortechnik AG, Switzerland). The residual solvent was further removed by hot air oven at 50 °C for 24 h.

Two-factor spherical composite experimental design

Two factors affecting the extraction of chemical constituent from *Centella asiatica* (L.) Urb. were investigated; extraction temperature (X_1) and extraction time (X_2). They have varied five levels from 40 to 60 °C and 60 to 120 min, respectively. The two-factor spherical composite experimental design was employed in this work. Ten extraction conditions were performed follow the experimental design; 8 non-center points and 2 center points as showed in Figure 1. The two responses including centelloids content (Y_1) and total phenolic content (Y_2) were monitored. The centelloids content and total phenolic content were estimated using Design-Expert® software version 11 (Stat-Ease, Inc., USA). Contour plots and response surfaces were constructed. Correlation between the predicted value and the actual value was investigated. The reliability and stability of the estimation were confirmed by the corresponding residual plot between internally studentized residuals and run number. The mathematic model, actual equation, and optimal condition of the estimation were also reported.



Run	Temp.		Time	
	Coded	Actual (°C)	Coded	Actual (min)
1	-1	42.9	-1	68.8
2	1	57.1	-1	68.8
3	-1	42.9	1	111.2
4	1	57.1	1	111.2
5	-√2	40	0	90
6	√2	60	0	90
7	0	50	-√2	60
8	0	50	√2	120
9	0	50	0	90
10	0	50	0	90

Figure 1. Two-factor spherical composite experimental design.

Determination of centelloids content

The 400 µg/mL of extract was prepared using methanol as solvent, filtered through syringe filter with pore size of 0.45 µm, and then injected into HPLC instrument (Agilent 1260 infinity, Agilent, USA). The content of individual centelloids was calculated from the calibration curve of madecassoside, asiaticoside, madecassic acid, and asiatic acid. The analysis was performed using HPLC equipped with photodiode array detector. Separation was done on ACE C18-PFP column (250×4.6 mm, i.d., 5 µm) with temperature controlled at 25 °C. Gradient elution was done use acetonitrile and 0.01% phosphoric acid aqueous solution (Table 1) with flow rate 1 mL/min. The injection volume was 20 µL and detection wavelength was 210 nm.

Table 1. The gradient system of HPLC analysis.

Time (min)	Acetonitrile (%v/v)	0.01% phosphoric acid (%v/v)
0.0	20.0	80.0
2.5	20.0	80.0
12	37.5	62.5
15	45.0	55.0
25	45.0	55.0
26	20.0	80.0
28	20.0	80.0

Determination of total phenolic content

The Folin-Ciocalteu assay was used for determination of total phenolic content (Slinkard and Singleton, 1997). The 0, 25, 50, 100, 150, and 200 µg/mL gallic acid aqueous solution was prepared and used as a reference standard. The 12.5 µL of the test sample or reference standard and 50 µL water was added to 96-well plate. The 12.5 µL Folin-Ciocalteu phenol reagent was added and the mixture was kept in the dark at room temperature for 6 min, followed by added 125 µL of 7% sodium bicarbonate and 100 µL water. The mixture was kept in the dark at room temperature for 90 min. The absorbance was measured at 760 nm using microplate reader (Benchmark Plus, Bio-Rad Laboratories, Inc., USA). The total phenolic content of plant extract was calculated from the calibration curve of gallic acid, which the unit of total phenolic content was mg gallic acid equivalent (mg GAE) per g dried weight.

DPPH radical scavenging assay

The 100-5,000 µg/mL of *C. asiatica* extract was prepared. The extract solution (n=3) was pipetted for 100 µL into 96-well plate. Then, the same volume of 0.2 mM of DPPH ethanolic solution was added and mixed together. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. The absorbance of the test sample (A_1) was compared to the absorbance of the control (A_0). The percent inhibition was calculated to follow Equation 1.

$$\% \text{ Inhibition} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100 \qquad \text{Eq.1}$$

The inhibition profile between percent inhibitions versus concentration of the extract was constructed. The half maximal inhibitory concentration (IC_{50}) was calculated from the equation of the inhibition profile. The IC_{50} value was used to compare to the centelloids and total phenolic content in the extract to investigating the linear correlation between IC_{50} value and content of each compound.

FRAP assay

FRAP reagent was prepared by mix 2.5 mL of 10 mM TPTZ with 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 300 mM acetate buffer pH 3.6. The mixture was incubated at 37 °C for 30 min, then cool to room temperature. The 100-5,000 µg/mL of *C. asiatica* extract was prepared. The assay was modified from Benzie and Strain (Benzie and Strain, 1996) and Wong et al. (Wong *et al.*, 2006). The extract solution (n=3) was pipetted for 30 µL into 96-well plate. Then, 270 µL of FRAP reagent was added. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 595 nm. The absorbance of the test sample (A_1) was compared to the absorbance of the control (A_0). The percent inhibition was calculated to follow Equation 2.

$$\% \text{ Inhibition} = \left(\frac{A_1 - A_0}{A_1}\right) \times 100 \qquad \text{Eq.2}$$

The inhibition profile between percent inhibitions versus concentration of the extract was constructed. The IC_{50} was calculated from the equation of the inhibition profile. The IC_{50} value was used to compare to the centelloids and total phenolic content in the extract to investigating the linear correlation between IC_{50} value and content of each compound.

Statistical analysis

The linear correlation between two data was studied using SPSS version 22 (IBM Corp., USA). Pearson correlation coefficient (R) was reported. Two data significantly correlated when *p*-value less than 0.05 at 95% confident interval.

RESULTS AND DISCUSSION

The centelloids content and total phenolic content obtained from ten model conditions could estimate the content of the two groups of compound using Design Expert® software. The contour plots and response surfaces of centelloids content in Figure 2 and 3 showed that the increase of extraction time, as well as extraction temperature, provided the higher centelloids content. In the case of extraction time, if the extraction system did not saturate, increasing of extraction time caused the solute more soluble. Increasing of extraction temperature could increase the solubility as well as diffusion coefficient of the solute (Dent *et al.*, 2013). However, the effect of extraction time and extraction temperature on centelloids content have not been reported previously.

In case of total phenolic content, at low extraction time, an increase of extraction temperature caused the higher content of total phenolic content. At the medium and high extraction time; an increase of extraction temperature, total phenolic content increased and then decreased. This result was similar to the increase of extraction time at low and medium extraction temperature. But, the increase of extraction time at high temperature provided the lower total phenolic content. Chew et al. studied the effect of extraction temperature and extraction time on the total phenolic content of *C. asiatica* that had a similar result to our work. They found that increasing of extraction time provided the lower total phenolic content due to higher oxidation of the compound. While, increasing of extraction temperature provided high total phenolic content (Chew *et al.*, 2011). Vergara-Salinas et al. found that high extraction temperature or high extraction time provided the lower content of polyphenols extracted from *Thymus vulgaris* (Vergara-Salinas *et al*., 2012). The extraction time had a positive effect on total phenolic content extracted from *Schizophyllum commune*. Conversely, extraction temperature had a negative effect on total phenolic content (Yim *et al.*, 2013). Dent et al. studied the effect of extraction temperature and extraction time on the total phenolic content of *Salvia officinalis* L., results showed that at 60 °C, the higher total phenolic content was found when compared to 90 °C. While, extraction time had no significant effect on total

phenolic content (Dent *et al.*, 2013). The previous publications indicated that extraction temperature had a positive effect on total phenolic content, while extraction time had several effects depend on plant type and specific extraction time used in each study. According to our work, the mathematic model of the estimation fitted with quadratic model. The actual equations for prediction of centelloids and total phenolic content are shown in Table 2.

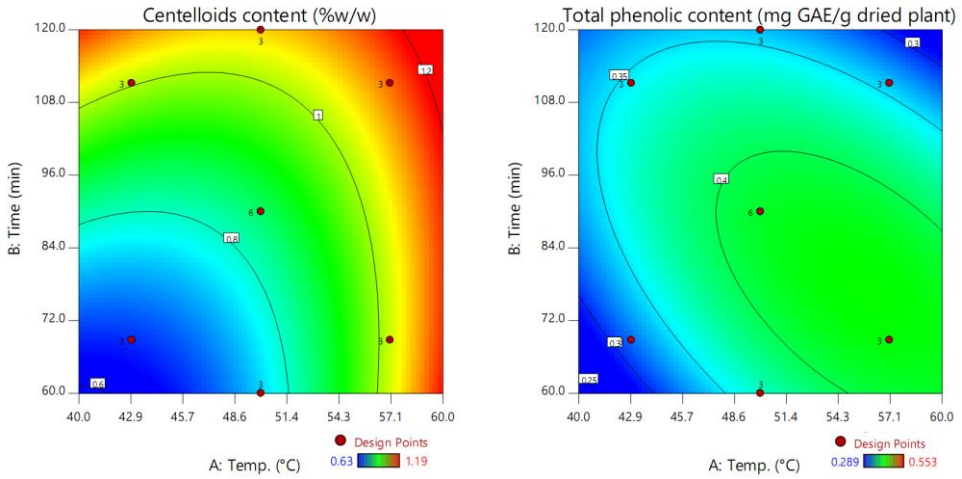


Figure 2. Contour plots of centelloids content (left) and total phenolic content (right) estimated by Design-Expert® software.

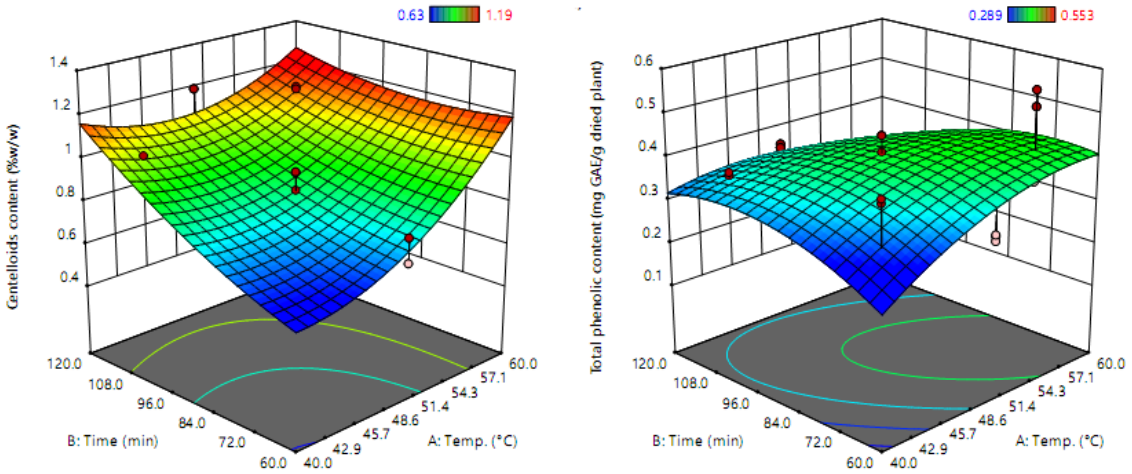


Figure 3. Response surfaces of centelloids content (left) and total phenolic content (right) estimated by Design-Expert® software.

Table 2. Model and the actual equation of centelloids and total phenolic content.

Response	Model	Actual equation
Centelloids content (Y_1)	Quadratic	$Y_1 = 1.69482-0.086924(X_1)+0.014452(X_2)-0.000411(X_1)(X_2)+0.001416(X_1)^2+0.000064(X_2)^2$
Total phenolic content (Y_2)	Quadratic	$Y_2 = -2.27061+0.068403(X_1)+0.020359(X_2)-0.000192(X_1)(X_2)-0.000481(X_1)^2-0.000063(X_2)^2$

X_1 = actual temperature, X_2 = actual time

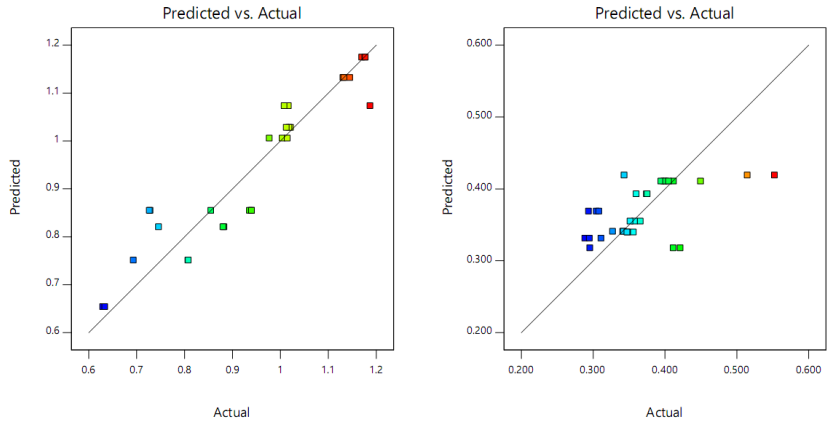


Figure 4. The linear correlation plots between the predicted value and actual value of centelloids content (left) and total phenolic content (right).

The reliability and stability of the estimation were confirmed by the linear correlation plots between the predicted value and actual value of two data (Duangjit *et al.*, 2014). The correlation coefficient was a parameter used to describe the linear relationship between two data. Figure 4 (left) showed the linear correlation plots between the predicted value and actual value of centelloids content. It had a high correlation coefficient (R = 0.9432) with *p*-value < 0.0001. Figure 4 (right) showed the linear correlation plots between the predicted value and actual value of total phenolic content. It had a low correlation coefficient (R = 0.5769). However, the *p*-value was 0.001, indicated that predicted value and the actual value was significantly linear correlated. These results could conclude that the estimation was reliable and stable.

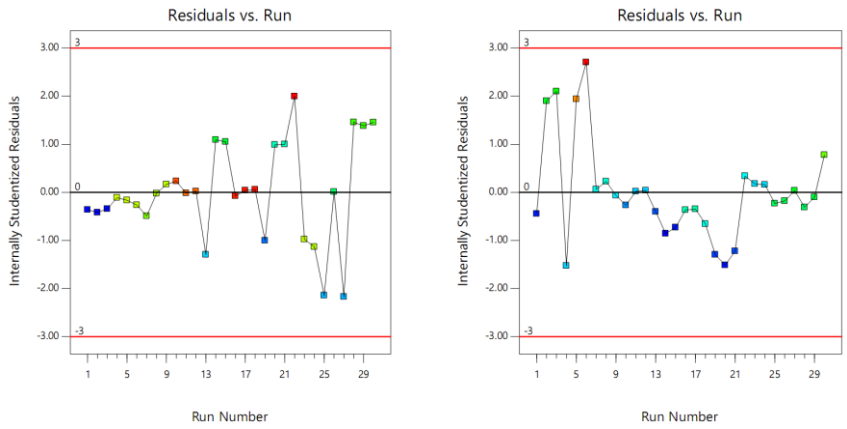


Figure 5. The corresponding residual plots between internally studentized residuals and run number of centelloids content (left) and total phenolic content (right).

The corresponding residual plots between internally studentized residuals and run number of centelloids content and total phenolic content showed the vertical spread of the internally studentized residuals after the randomized run was within the red line (Figure 5). This result indicated that all points were within the limit at 95% confident interval.

The optimal conditions provided from the estimation for centelloids and total phenolic content were 59 °C and 108.1 min with the desirability of 1.000, and 55.7 °C and 77.4 min with the desirability of 0.505, respectively. The centelloids and total phenolic content estimated from the optimal condition were 1.256 %w/w and 0.422 mg GAE/g dried plant, respectively.

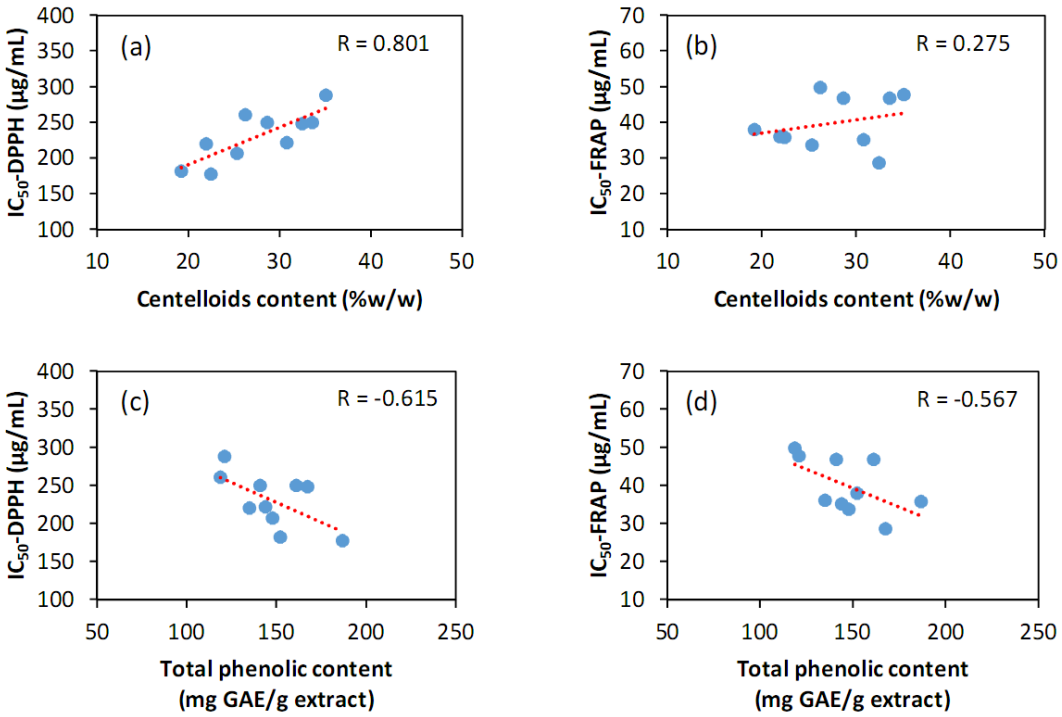


Figure 6. The linear correlation between centelloids content (a, b) or total phenolic content (c, d) and IC_{50} obtained from DPPH (left) and FRAP (right) assay.

The linear correlation study found the significant result only when to compare between centelloids content and IC_{50} obtained from DPPH radical scavenging assay (*p*-value = 0.005). While, the other comparisons were not significant correlated; *p*-value of comparison between centelloids content and IC_{50} obtained from FRAP assay, total phenolic content and IC_{50} obtained from DPPH radical scavenging assay, and total phenolic content and IC_{50} obtained from FRAP assay were 0.443, 0.058, and 0.088, respectively. However, Figure 6a and 6b showed that when centelloids content increased, IC_{50} also increased due to low antioxidant activity of centelloids; the content of another antioxidant might be low in the extract with higher centelloids content. However, there was some report showed that some centelloids could exhibit as in vivo antioxidant i.e. asiaticoside (Shukla, Rasik and Dhawan, 1999; Shukla, Rasik, Jain, *et al.*, 1999) and madecassoside (Liu *et al.*, 2008). According to Figure 6c and 6d, when total phenolic content increased, IC_{50} decreased because of the potent antioxidation property of phenolic compound (Rice-Evans *et al.*, 1997). These results indicated centelloids had a negative effect, while total phenolic content had a positive effect on antioxidant activity. In this case, the authors mentioned the complexity of the chemical constituent of the herbal extract, no individual active chemical constituent was recognized as a major contributor to overall antioxidant activity (Vassallo, 2008).

CONCLUSION

Centelloids and total phenolic content were estimated using computer software in this work. The effect of two factors; extraction temperature and extraction time were investigated. The estimation equation of both groups of compound fitted with quadratic model. The estimation was reliable and stable. The optimal conditions that provided the highest centelloids and total phenolic content were 59 °C vs 108.1 min and 55.7 °C vs 77.4 min, respectively. Furthermore, centelloids content had a negative effect on antioxidant activity from DPPH radical scavenging assay. But, no correlation was found from FRAP assay. There

was no correlation between total phenolic content and antioxidant activity from DPPH radical scavenging assay as well as FRAP assay. In addition, this extraction condition that provided the highest content of each compound were used as standard condition for extraction of *C. asiatica* in our further work.

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