Antioxidants, Polyphenols and Marker Phytochemicals Content from Different Parts of *Clinacanthus nutans* (*C.nutans*)

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ABSTRACT *Clinacanthus nutans (C.nutans) or* Belalai Gajah has become popular medicinal herb for Malaysian community due to worthy biological property. It has been used in widespread of food products such as tea and supplements. The present study was focussed the determination of phytochemical components of *C.nutans* of the three different plant parts consists of leaves, stem and plant mixture. The experiments deal with the assessment of antioxidant property, total phenolic content and marker phytochemicals (orientin and vitexin). Free radical scavenging activity based on DPPH and ABTS assays were carried out for the antioxidant property. Meanwhile, the phenol content and identification of marker phytochemical were measured by using Folin-Ciocalteu's method and HPLC analysis respectively. Leaves samples exhibits stronger scavenger ability (DPPH, $953.22\pm 3.49_a$ µg/ml and ABTS,$1693.33\pm0.23_a$ µg/ml) compared to other samples. The total phenols content and both marker phytochemical of orientin and vitexin were found higher in leaves among the samples. Stem indicated the least results among the parameters tested. The total phenolic content in the leaves was 125.83 ± 0(mg GAE/g) while orientin represented 0.1235, w/w% and vitexin represented 0.1421 w/w%, respectively. The present results indicated leaves from *C.nutans* have higher chemical property and could be preferable part to be used for further phytochemicals research.

KEYWORDS: *Clinacanthus nutans*; polyphenols; antioxidant property; vitexin; isovitexin.

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INTRODUCTION

Belalai gajah or its scientific name ; *Clinacanthus nutans, (C.nutans*) is a herbal plant can be found throughout South East Asia region, especially in Thailand and Malaysia. This shrub plant belongs to the acanthaceae family, and locally known in Malaysia for Malay community as Sabah snake grass. The plant criteria small, thin and slightly curved stem that resembles the curve of an elephant trunk (Roosite *et al.*, 2008). In other county such Thailand, it is known as phaya yo or phaya plongtong which was described in a literature by Smitinand (1980). Traditional practice highlighted this plant for anti-snake venom, skin rashes as well as insect bites (Sakdarat *et al.*, 2006).

Scientific evidence showed that this plant served several biological activities that were contributed due to variations of phytochemical constituents including cerebrosides, monoacylmonogalactosylglycerol, flavones and chlorophyll derivatives (Sakdarat *et al.*, 2009; Chelyn *et al.*, 2014). Moreover, the medicinal properties such as antioxidant, anticancer as well as anti-inflammatory have been reported in this plant, also from the leaves (Tuntiwachwuttikul, 2004; Sakdarat *et al.*, 2006, P'ng *et al.*, 2012). In addition, the flavone derivatives consists of vitexin, isovitexin, shaftoside and orientin are types of known marker phytochemical reported in *C.nutans* leaves, served numerous biological properties such as antimicrobial activity, hepatoprotective activity and antioxidant activity (Zhang *et al.*, 2008; Huang *et al.*, 2012).

Lacking of phytochemical studies had received more attention to scientist to provide reliable information of this herb. However, recent studies are mainly focussed on the leaves, rather than the other plant part. This is regards to its rich in biological properties in the leaves part. The study will be covered the aspect of phytochemical of *C.nutans* from three different plant parts; leaves, stem and mixture of stem and leaves. Phytochemical analysis including assessment of antioxidant property, polyphenols content as well as markers phytochemical by chromatographic analysis (vitexin and orientin) will be further investigated.

METHODOLOGY

Plant material: Clinacanthus nutans

C.nutans samples of leaves, stem, mixture sample (leaves+stem) were taken from Nottingham University, Semenyih, Selangor, Malaysia. The samples were freshly harvested at the age of maturity, clean and separated into three different plant parts of leaf, stem and a portion of stem and leaves mixtures (randomly). Samples were dried by using an industrial solar dryer built at the Pusat Pembangunan Komoditi Sendayan, Negeri Sembilan, Malaysia.

Chemical and standards

The standard compounds of vitexin and orientin were purchased from Sigma-Adrich (St. Louis, MO). Gallic acid and ascorbic acid were supplied from Across organic (Pittburgh, P.A). The antioxidant chemicals procured Sigma-Adrich (St. Louis, MO). Methanol and acetonitrile (HPLC grade) were supplied from Merck (Co., Darmstadt, Germany). Meanwhile, methanol and ethanol (reagent grade) from Qrec, (Republic of New Zealand). Folin-Ciocalteu's and sodium carbonate was from Sigma-Adrich (St. Louis, MO). Ortho-phosphoric acid from Merck (Co., Darmstadt, Germany).

Methanolic extraction of Clinacanthus nutans

Samples were crumpled and separated plant parts were grind using warring blender (particle size <200 micron). Plant material (6 gram) was extracted with 70% methanol:water (60ml). Sample boiled before refluxed at 1 hour and cooling for half an hour before filtered using (smith, code 125 mm). Then, the filtrate solutions were evaporated (Heidolph, Germany) until the volume concentrated quarters of its initial volume before poured into petri dish and drying overnight at 50 °C temperature. The dried or crude extracts were stored (-25C temperature) prior for analysis.

Antioxidant assays: DPPH

The DPPH assay was developed according to the published method by Norliza *et al.*, (2016). Sample and DPPH was stored under dark place until completed chemical reaction in 30 minutes before measured the decreased of absorbance that was monitored at 517 nm using UV Spectrophotometer UV-1800 (Shimadzu Corporation, Japan). All determinations were carried out in triplicates. Ascorbic acid was used as standard reference. All determinations were carried out in triplicates. DPPH scavenging activity (%) was calculated using the equation below:

$$DPPH\ scavenging\ activity\ (\%) = \frac{(A_0 - A_1)}{A_0}\ x\ 100 \quad (1)$$

Where,

A_0 = Absorbance of control

A_1 = Absorbance of sample

Antioxidant assays- ABTS

The method for this assay was adapted from Norliza *et al.*, (2016) that modified from Re *et al.*, (1999). Firstly, ABTS•+ solution was prepared after 16 hours of incubation time, and it was diluted

with distilled water to get the final absorbance of 0.700 at 734 nm using UV-visible spectrophotometer (Shimadzu Corporation, Japan). 1300 µl of the ABTS•⁺ solution was mixed with 200 µl of a plant extract before incubated in the dark place for 10 min at room temperature and subsequently measured. Equation 1 was used to calculate the ABTS•+ radical scavenging activity (%). Ascorbic acid was used as a reference standard.

Total polyphenols content

Folin-Ciocalteu's assay was used to determine the total phenolic content, and the procedure was according to the Norliza *et al.*, (2016). After 2 hours incubation time, the absorbance was recorded at 765 nm using UV-visible spectrophotometer (Shimadzu Corporation, Japan). Gallic acid was used as reference standard. Graph of calibration curve was plotted and results were expressed as Gallic acid equivalent (mg GAE/100 g DW).

Chromatography analysis by HPLC

HPLC-UV/DAD analysis was performed on a Waters e2695 Separations module (Millford, MA, USA) connected with photo diode array (PDA) detector. The detection method was referred from Chelyn *et al.*, (2014) with slight modifications. The separation of UV chromatographic was performed using X bridge column (250 mm in length, internal diameter 4.6mm, 5 µm particle size, Waters, Millford, MA). Mobile phase A was prepared in dH_2O+0.1 ortho-phosphoric acid, and mobile phase B in 100% acetonitrile (HPLC grade). The system was optimized with A: B (95:5) at flow rate of 0.8 ml/min using isocratic in a 20 minutes. Injection volume was 20 µl and the signal monitor at 300-350 nm using Empower 3 Software 2010. Subsequently, crude samples (0.1g) were taken out and diluted with 10mL methanol. Samples were then filtered using 0.45 µm syringe filter prior HPLC analysis. The standards stocks (1.5 mg in 6 ml) of vitexin and orientin were mixed for chromatographic detection, which were diluted in five different concentrations. The quantitative results were expresses as 1 mg of compound in 1 g extract (% w/w).

Statistical analysis

The experiments were carried out by three measurements and expressed as the mean of reading ± standard of deviation. Statistical approach of SPSS version 16.0, was performed to compare means in one-way analysis of variance (ANOVA) and p value set at <0.05 (5% probability value) subjected to statistically significantly differences among the variables tested.

RESULT AND DISCUSSION

Antioxidant property of different plant parts

Antioxidant activity on leaves, stem and plant mixture were assayed based free radical scavenging assays. Table 1 represented results that obtained from effective concentration (IC_{50}). Antioxidant property by DPPH assay showed that the IC_{50} of leaves is slightly higher among the samples. Meanwhile, the IC50 for ascorbic acid was comparable in both assays. Results from ABTS assay indicated similar order as presented in Table 1. Therefore, both antioxidant assays had proved that leaves was the best plant part that rich in antioxidant activity. Many studied was emphasized that leaves has more ability to produce more antioxidant compound due to the polyphenols. For instance, *C.nutans* was reported to rich in polyphenols components (Chelyn *et al.*, 2014; Zhang *et al.*, 2008; Huang *et al.*, 2012).

There are different studies that indicated *C.nutans* leaves extract obtained from chloroform as the main solvent was reported to produce high antioxidant property by DPPH assay when it was

compared to the water and methanol extracts (Yong *et al.*, 2013). Similarly, *C.nutans* ethyl acetate and dichloromethane leaf extracts had also contain high antioxidant activity using DPPH assay (Sulaiman *et al.*, 2015). However, their results cannot be concluded because they only measured the antioxidant activity based on single assay. Comparative antioxidant assay should be carried out to conclude the results. This is because; antioxidant compound could be hydrophilic or lipophilic characters. The action antioxidant is likely complex and depending on the system itself (mechanism) to react with the free radicals. However, solvent types may play role with the antioxidant mechanism and the results varied as subject to energy that produced by radical to make a reaction with antioxidant components. DPPH assay is applicable in lipophilic character; meanwhile ABTS assay can react with hydrophilic or lipophilic characters (Apax *et al.*, 2013, Alam *et al.*, 2013). Antioxidant by free radical scavenging of DPPH and ABTS are comparatively straightforward assays and combining both techniques may produce better results (Alam *et al.*, 2013).

Table 1. The extraction yield and antioxidant activity of *C.nutans*

Samples	**Antioxidant property**		**Total phenolic content**
	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	
Leaves	$953.22 \pm 3.49_a$	$1693.33 \pm 0.23_a$	$125.83 \pm 0_e$
Stem	$2629.63 \pm 5.77_b$	$1745.93 \pm 1.20_b$	$100.74 \pm 1.4_f$
Mix (leaves + Stem)	$3915.4 \pm 11.35_c$	$1970.10 \pm 2.51_c$	$66.83 \pm 0_g$
Ascorbic acid	$26.13 \pm 2.43_d$	$25.55 \pm 5.34_d$	-

Data are expressed as mean ± SE (n = 3). Mean with different letters in a column differ significantly at p<0.05 subject to different type of samples.

Total polyphenols content on the plant parts

Total polyphenols of *C.nutans* plant parts was spectrophometrically measured at 716 nm wavelength. Results as mentioned in Table 1 revealed the phenolic content among samples are varied, ranging 66.83 and 125.8 (mg GAE/g). The extensive reduction of Folin-Ciocalteu's reagent from yellow to blue colour indicated leaves higher in phenolic content followed stem and mixture samples. Moreover, the study seems to be consistent with the other group of researchers who reported that the fermented *C. nutans* tea contains high amounts of phenolic that exhibits powerful antioxidant activity (Dufresne *et al.*, 2001).

HPLC analysis was performed in an isocratic system to elute the marker of interest in quantitative chromatographic system and separation results were acquired within 18 minutes. In this study, two markers phytochemical namely vitexin and orientin in the *C.nutans* was investigated. This is in fact that vitexin and orientin have displayed a great potential for medicinal purpose and that effect such as anticancer and antioxidants has been discussed in many journals (Yang et al., 2011). In this current work, the identification and quantification of vitexin and orientin were performed and an adequate separation of known chemical markers was achieved with another four significant peaks by constant flow rate in isocratic system. As results, the chromatograms were shown in Figures 2.

This study observed the first chemical phytochemical marker, orientin which was eluted at 11.7 minutes; meanwhile vitexin was eluted at 12.6 minutes. Significantly, there were several types of compounds in *C.nutans* and some of it had been identified in the previous study. Peak labelled as 1, 2 and 3 represented the C-glycoside type's flavonoids; shaftoside, isoorientin and isovitexin, and three other unknown compounds that matched well with the previous study (Chelyn *et al.*, 2012). Interestingly, the unknown peak iii showed the higher intensity after peak 1, however it has not been discovered in any literature. To compare, orientin was eluted at 22.58 minutes and vitexin at

28.61 minutes (Chelyn *et al.*, 2012). This study was consistently separated these two compounds within 18 minutes run time. Thus, fast separation system was achieved in this present study.

Concentration of orientin falls from the lower value to higher value; between 0.0064 % and 0.124%. Meanwhile, vitexin was determined between 0.004 to 0.142%. Leaves presented the most concentration of vitexin and orientin; 0.124%, and 0.142% respectively. While, the least concentration was determined in stem samples. This study showed that leaves presented the most abundant content of vitexin and orientin compared to other parts of plants. The presence of these components is probably contributed with its medicinal function in this herb. This is in good agreement with those whom reported the presence of orientin and vitexin are plays an important role with the antioxidants effect toward various medicinal properties (Yang *et al.*, 2011).

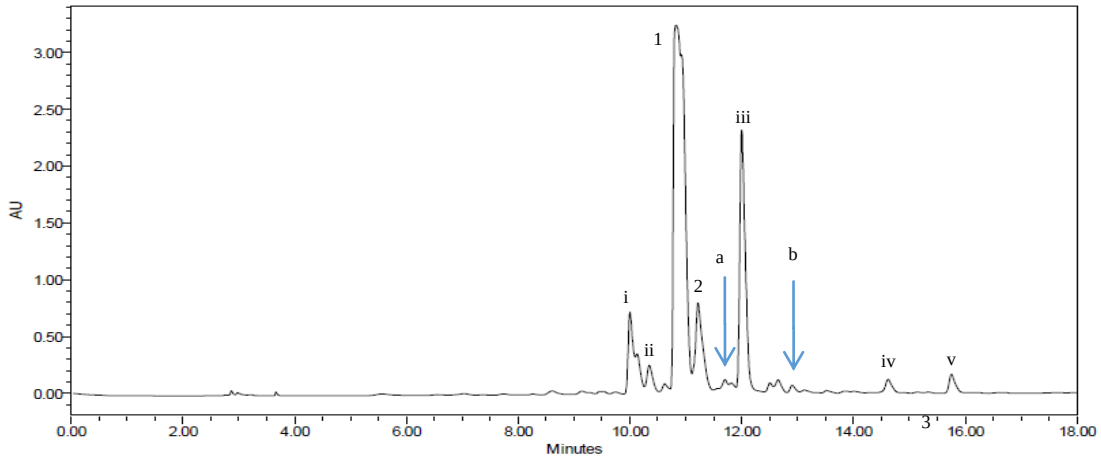

Figure 2. HPLC chromatogram obtained from *C.nutans* leaves part; a (orientin),b(vitexin),i-v(unknown), The chromatogram and sequenced of peak 1-3 and i-ii and iv-v were matched as reported by Chelyn *et al.*, 2012, represented shaftoside, isoorientin and isovitexin respectively. Peak iii was the unique unknown peak and has not been reported.

CONCLUSION

This study concludes that leaves could be the most important plant part in *C.nutans* attributed with the highest extraction yield, phenolic content and antioxidant property. It is presumed the higher amount of phenolic in the leaves is due to the presence of orientin and vitexin as chemical marker. The study suggested that leaves may be useful for medicinal and nutritional purposed. However, further investigation is proposed to isolate and characterized the other phytochemicals component from the leaves as many peak has not been discovered in any literature.

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