TOTAL PHENOLIC CONTENT AND THE ANTIOXIDANT ACTIVITY OF *CLINACANTHUS NUTANS* EXTRACT

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ABSTRACT

Introduction: *Clinacanthus nutans* is used as natural nutraceuticals for prevention and treatment of cancer. The purpose of this study is to (i) determine the total phenolic content and antioxidant scavenging capacities of *C. nutans* in free and bound phenolic acid and (ii) study the relationship between TPC and antioxidant scavenging capacities of *C. nutans*. **Methods:** The total phenolic contents were measured using Folin-Ciocalteu assay. Free and bound phenolic were examined by using spectrophotometer while antioxidant capacity were evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging activity assay. **Results:** Insoluble phenolic acids showed the highest amount of total phenolic content in *C. nutans* extracts (6.09± 0.45 mg gallic acid equivalent (GAE)/ g DW) and exhibited highest antioxidant activity (73.3±0.82 %) as compared to free and soluble phenolic extracts. The IC_{50} values for free phenolic, soluble bound and insoluble bound phenolic extracts were 0.69±0.02 mg/mL, 0.64±0.04 and 0.60±0.006 mg/mL, respectively. There were positive correlation between insoluble bound phenolic content of *C. nutans* extracts with antioxidant radical scavenging capacity (R^2 = 0.893). **Conclusions:** These results indicate that different phenolic acid forms affect the total phenolic content and antioxidant properties. Natural compounds such as phenolics from *C. nutans* could be a good source of antioxidant.

Keywords: *Clinacanthus nutans* , TPC, DPPH scavenging activity assay

INTRODUCTION

In Malaysia, more than 3000 types of medicinal plants are used as traditional medicine. The use of *Clinacanthus nutans* as natural nutraceuticals for prevention and treatment of cancer has increased popularity in Malaysia (Yong et al. 2013; P'ng et al. 2013). This herb is traditionally believed to cure nettle rash, dysentery, heals burns, scalds, insect stings and fever (P'ng et al. 2013). *C. nutans* is commonly used in medication due to its antioxidant properties (Yahaya et al., 2015) and various phytochemical compounds found in the extracts (Tu et al. 2014).

Clinacanthus nutans (Burm. f.) Lindau which originates from tropical Asian region is locally known as 'Sabah snake grass 'or 'Belalai Gajah' in Malaysia and generally used to treat various diseases (Lusia et al. 2015; Raya et al. 2015). Many studies were done on the traditional medicine and pharmacological activities of *C. nutans*. It is effective in the recovery from insect and snake bites and for the treatment of skin rash. The leaves of *C. nutans* are able to treat herpes simplex virus (HSV), varicella - zoster virus (VZV) lesions and viral infection (Chelyn et al. 2014; Yong et al. 2013; Sakdarat et al. 2009). It has been proven to have antioxidant, anti-diabetic, anti-inflammatory, analgesic, anti-rheumatism, anti- venom and anti-viral properties (Raya et al. 2015; P'ng et al. 2013; Yahaya et al. 2015). Phytochemicals are able to quench free radicals and act as antioxidants against oxidative chain propagation (Garcia-Salas et al. 2010). Phytochemicals that are found in abundant in plants are phenolic compounds (Zlotek et al., 2015). Phenolic acids are present in two forms which are free and bound phenolics (Khoddami et al. 2013). Cereal kernels for instance, contain phenolic acids and are found in a free and bound form (Abdel-Aal et al. 2012). Phenolics compound from plants extraction are usually found in glycoside conjugate and insoluble forms but are hardly found in free forms of phenolic(Dai & Mumper, 2010). Previous studies have focused on total phenolic content in free phenolic parts (Roby et al.; Li et al. 2009). Little information reported on free and bound forms of phenolic acids compounds in *C. nutans* in Malaysia. Thus, this study was conducted to determine the total phenolic content in both free and bound extract and to study its relationship with antioxidant scavenging capacities.

METHODOLOGY

Collection of *C. nutans* plants

C. nutans plants were collected from Terengganu, Malaysia. The plant leaves were washed thoroughly under running tap water and kept in -80 °C freezer for one day before being dried in a freeze dryer for one week. The dried plants were ground to fine powder by using a blender at room temperature.

Free Phenolic Extraction

The free phenolic extraction method was described by Singh et al. (2013) with some modifications. The experiments were done in two batches and the samples were prepared in three replications. The dried plant material (1g) was mixed with 20 ml of 80% methanol. The samples were mixed with a sonicator at room temperature for 60 minutes. After that, the supernatant were taken out by using Whatman filter paper No.1. The residue pellet was kept for further analysis on bound insoluble phenolic acids. The steps were repeated twice. The supernatant were desolventised in the rotary evaporator until semisolid residue was obtained. The residue was then extracted by washing it with diethyl ether for three times and the organic phase was collected (contain free phenolic acids). The organic solvent was evaporated until all the solvent were removed (pressure 850 mbar). The dry residue was mixed with 10 ml of methanol and was labelled as free phenolic acid and kept under 4°C for further analysis. The aqueous phase was kept for further analysis of bound-soluble phenolic acids.

Bound Soluble Phenolic Extraction

The soluble phenolic extraction method was described by Singh et al. (2013) with some modifications. Thirty millilitre of 4 M NaOH was mixed with the residue pellet and incubated at room temperature for two hours. The pH was adjusted to pH 2 with 6N HCl. Then, the sample was extracted with diethyl ether for three times and the organic phase was collected which contain bound soluble phenolic acids. The organic extract was evaporated to dryness. The dried residue was dissolved in 10 ml of methanol and was labelled as soluble phenolic acid and kept under 4°C for further analysis.

Bound Insoluble Phenolic Extraction

Bound insoluble phenolic extraction method was described by Abdel-Aal, et al. (2012) with a slight modification. The residual pellet obtained was mixed with 20 ml of 4 M NaOH at room temperature for one hour. Then, 6 N HCl was added in the mixture until pH 2 was obtained followed by centrifugation (4300 rpm, 10 min). The liberated phenolics were extracted with 15ml hexane at a hexane to water ratio (1:1 v/v) to remove fatty acids and other lipids contaminants (Singh et al.2013) with some modifications. The aqueous layer was extracted three times with diethyl ether and the organic phase collected was evaporated to dryness. The dried residue was dissolved in 10 ml of methanol and labelled as insoluble phenolic acid and kept under 4°C for further analysis.

Folin – Ciocalteu's Assay

Folin – Ciocalteu's method described by Ismail et al. (2010) was chosen to determine the total phenolic content with a slight modification. The concentration of each sample extracts was prepared at 1mg/ml. Ten different concentrations of sample/standard were prepared in the range from 0.01 to 0.1 mg/ml. Sample or standard (gallic acid) or blank (distilled water) with 150 µl volume for each was transferred into a cuvette. Then, 2.5 ml of the Folin-Ciocalteu reagent was added to the solution. The mixtures were incubated for 5 minutes at room temperature. Two millilitres of 5% (w/v) sodium carbonate was added and gently mixed. The reaction mixtures were incubated for about 60 minutes at room temperature. By using UV/Vis spectrophotometer, the mixtures were measured at 725 nm. Finally, the standard calibration curve of gallic acid was plotted between concentration 0.01 to 0.1 mg/ml. The analyses were done in triplicate and the results were expressed as milligrams Gallic Acid Equivalents per gram of extract weight (mg GAE/ g extract). The equation below was used to calculate total phenolic content of extracts:

$$T = C \times V/M$$

Where, T = total phenolic content (mg/g) of the extracts as GAE, C = concentration of gallic acid (mg/ml) from the calibration curve, V = volume (mL) of sample and M = weight (g) of the extract.

DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) assay

The DPPH scavenging capacity was conducted by using an assay as described by Ravishankar et al. (2013) with slightly modifications. The standard used was gallic acid and distilled water was used as blank. Stock solution (64 mg/l) of gallic acid was diluted with distilled water in 10 different concentrations ranged from 0.5 to 64.0 mg/l. The sample extracts were prepared in 5 different concentrations (dilution factor of 1:1, 1:5, 1:10, 1:15, 1:20) and were diluted with distilled water. Then, 500 µl of sample were added with 2.0 ml of 0.079% of DPPH solution in the 96 well microplates and incubated in the dark for 30 minutes. By using microplate reader at 517nm, the absorbance of the solution was measured. The percentage (%) inhibition of radical scavenging activity was calculated using the following formula:

$$\text{DPPH Radical Scavenging Activity (\%)} = \frac{A - B}{A} \times 100$$

Where, A = absorbance reading of blank and B = absorbance reading of sample.

IC_{50} values were calculated to find the samples concentration required to reduce 50% absorbance of DPPH by plotting graph of percentage inhibition of DPPH scavenging activity against samples concentration.

Statistical Analysis

All experimental values were measured in triplicates (n=3) and expressed as means ± standard deviations (SD). All data were analysed using Excel (2010) and SPSS software version 23. One way analysis of variance (ANOVA) was test to determine the different mean in *C. nutans* extracts. Pearson's linear correlation test was utilized to analyse the correlation between phenolic compound and antioxidant activity. The confidence limits (p-values) were set at $p < 0.05$.

RESULTS

Total Phenolic Content of *C. nutans*

Total phenolic content (TPC) of *C. nutans* was extracted into three parts which were free, bound soluble and bound insoluble phenolic. Determination of phenolic content in different forms was analysed by using Folin-Ciocalteu (FC) colorimetric assay. TPC is a qualitative measurement to determine the concentration of phenolic by performing a calibration curve. A linear calibration curve of gallic acid standard was obtained from 10 concentrations of gallic acid ranged from 0.01 mg/ml to 0.1 mg/l (y=3.1018x and R^2 = 0.9982). The results were expressed as milligrams Gallic Acid Equivalents per gram of dry weight (mg GAE/ g DW). The Figure 1 shows that the highest TPC was insoluble phenolic as it forms high intensity of blue colour compared to other phenolic acids.

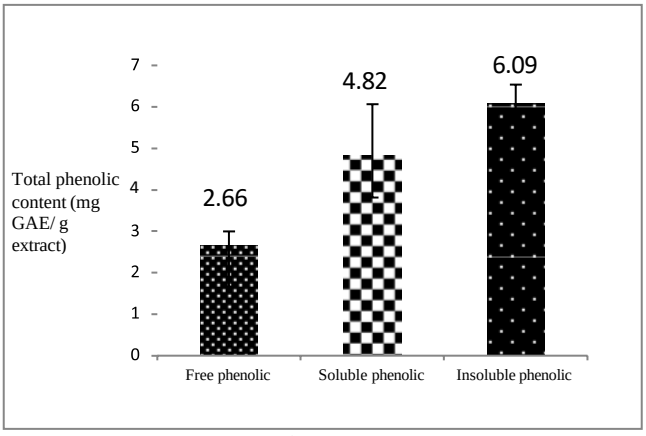

Figure 1
Total phenolic content of different *C. nutans* extraction

The highest value of TPC was observed in the insoluble phenolic extract (6.09± 0.4471 mg GAE/ g DW) followed by soluble bound phenolic and free phenolic extract (4.82±1.2488 mg GAE/ g DW and 2.66±0.3301 mg GAE/ g DW, respectively) (p<0.05). TPC of *C. nutans* can be ranked as follows: insoluble bound > soluble bound > free phenolic extracts. The significant difference (p<0.05) between free, soluble and insoluble phenolic have been observed through one way analysis of variance (ANOVA) and post hoc comparisons using Tukey's test. One way (ANOVA) illustrated that the mean difference of phenolic form (p<0.05) affect the TPC value of each extract. Meanwhile, the comparisons in Turkey's test demonstrated that there was a significance difference between free and soluble; free and insoluble. However, there is no significant difference (p< 0.05) between soluble and insoluble phenolic. Thus, it can be concluded that the different form of phenolic acids influenced the amount of TPC in plant extracts.

Antioxidant Activity of *C. nutans* Extracts Using DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Assay

Different types of phenolic extract were examined to observe their antioxidant radical scavenging capacity.

A standard curve of gallic acid standard (R^2= 0.9989) was calibrated in eight different concentrations ranged from 0.5 mg/l to 64 mg/l of gallic acid. The absorbance of each sample was measured at 517 nm and gallic acid act as a positive control. The radical scavenging capacity is directly proportional to the concentration of extracts. Figure 2 illustrated the percentage of radical scavenging capacity of each type of phenolic acids in five concentrations (DF 1:1, 1:5, 1:10, 1:15, and 1:20). It shows that the highest percentage of radical scavenging capacity for every phenolic acid form was at concentration 1 mg/L (DF 1:1). At this concentration (DF = 1), the insoluble bound extract showed the highest percentage of radical scavenging (73.3±0.82 %) as compared to the soluble bound extract (72.7+1.31 %) and free phenolic extract (68.0±3.32 %) (p<0.05). The antioxidant scavenging capacity can be ranked as follows: insoluble> soluble> free phenolic.

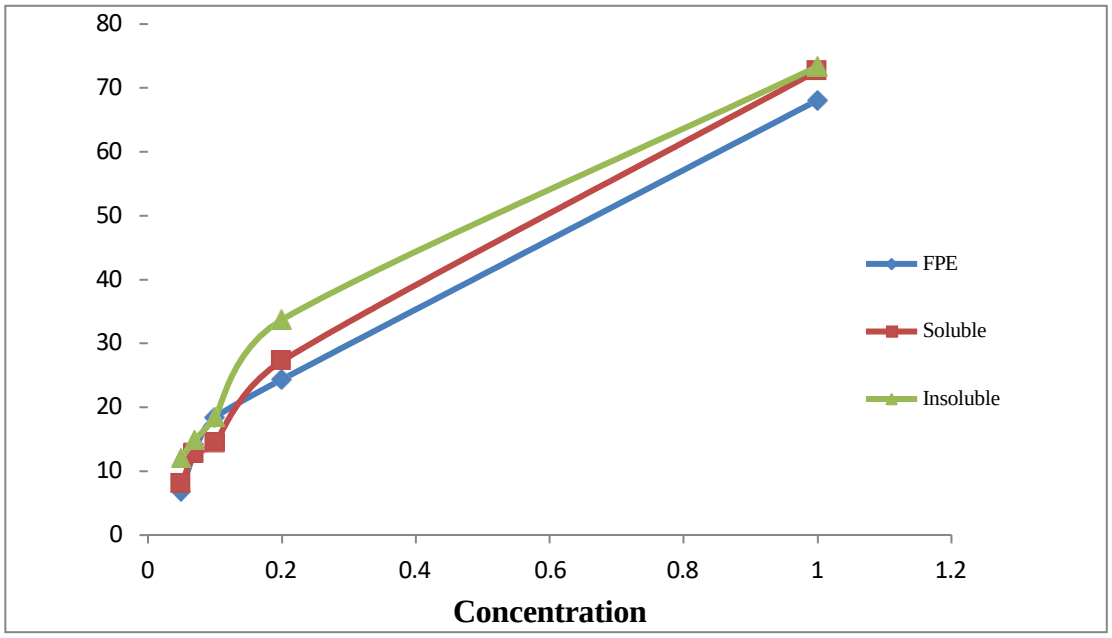

Figure 2
Scavenging capacity of free, soluble and insoluble extracts on DPPH radicals

The efficacies of these extracts as antioxidants were measured and expressed as IC_{50} value. The IC_{50} is inhibition concentration of antioxidant which reduces 50% of free radical DPPH. The IC_{50} is inversely proportional to radical scavenging capacity. Table 1 shows the value of IC_{50} in insoluble bound, soluble bound and free phenolic acids extracts. Lowest amount of IC_{50} in the insoluble bound phenolic acids implies strong reducing power which contain higher amount of radical scavenging capacity.

Table 1 IC_{50} values of free, soluble and insoluble extracts in DPPH assay

Samples	IC_{50} (mg/mL)
Free phenolic extract	0.69±0.02*
Soluble bound phenolic extract	0.64±0.04*
Insoluble bound phenolic extract	0.60±0.006*

Each value is the mean from three analyses ± standard deviation. * Indicates significant different at $p<0.05$.

One way analysis of variance (ANOVA) conducted by using Excel 2010 demonstrated that there is significant difference between each extract of *C. nutans* and antioxidant inhibition. Thus, it can be concluded that different forms of phenolic affect the radical scavenging capacity and inhibition concentration. Post hoc comparisons using Turkey's test examined the significant difference between free, soluble and insoluble bound phenolic. From multiple comparisons test, the results indicated that free and insoluble bound phenolic were statistically significant difference ($p<0.05$). Thus, it can be concluded that the free and insoluble bound phenolic extracts affected the antioxidant radical scavenging capacities of the extracts. However, there are no significant differences observed between free and insoluble bound phenolic; soluble with insoluble bound phenolic.

Correlation between Total Phenolic Content and Antioxidant Radical Scavenging Capacity *C. nutans* Extracts

The correlation between antioxidant activities and TPC in different extracts can be analysed quantitatively. Therefore, the Pearson's linear correlation test and linear regressions were used to determine the relationship between TPC (free, soluble and insoluble bound phenolic) and antioxidant radical scavenging capacity. The strongest positive correlation was observed between insoluble bound phenolic and antioxidant radical scavenging capacity ($R^2= 0.8926$) with free phenolic ($R^2= 0.8247$). There is no significant difference found between TPC (free and insoluble bound phenolic) and antioxidant radical scavenging capacity ($p<0.05$).

There was moderate negative correlation between soluble bound phenolic and antioxidant radical scavenging capacity (R= -0.6392). From Pearson's linear correlation, there is no significant difference ($p<0.05$) between this correlation. The results obtained show the soluble bound phenolic extract is high with TPC but low in antioxidant radical scavenging capacity.

DISCUSSIONS

Total Phenolic Content of *C. nutans*

The FC reagent used in this assay measures total reduction capacity of extract in all compounds not specific to phenol only (Ikawa et al. 2003). The change of colour of FC reagent from yellow to blue indicates that oxidation reduction reaction has occurred (Azlim Almey et al. 2010; Ruzlan et al. 2010). High intensity of blue colour during that reaction indicates the amount of polyphenols concentration in the extracts. TPC of *C. nutans* can be ranked as follows: insoluble bound > soluble bound > free phenolic extracts. Previous study by Singh et al. (2013) demonstrated that phenolic content in bound phenolic (soluble and insoluble) of *Moringa oleifera* leaf was higher (45.81 ± 0.02 mg GAE/100g) as compared to free phenolic (36.02 ± 0.01

mg GAE/100g) ($p<0.05$). The present study is in line with Durdevic et al. (2013), who reported that bound phenolic acids (soluble and insoluble) contain 3.24± 0.29 mg/g dry weight of TPC value higher than free phenolic extract (1.10±0.17mg/g dry weight) in *Allium ursinum* extract. Canini et al. (2007) also proved that extraction of *Carica papaya* L. leaf exhibited high phenolic concentration when hydrolysed with acid as compared to non-hydrolysed extract particularly for caffeic, p-coumaric and protocatechuic acids. These results indicated most of the phenolic compounds are in bound form either esters or glycosides forms. Thus, the results in this present study is consistent with the finding by Germano et al. (2006) which discovered that *Trichilia emetic* (Natal-Mahogany) extract consisting mostly of bound phenolic and only a small amount of free phenolic compound. It can be concluded that bound phenolics (soluble and insoluble) were predominant over free phenolic in different types of plants.

Different types of plant have different amount of TPC due to the different composition of chemical and polyphenol compounds that are present in each plant (Blainski et al. 2013). The extraction of phenolic compounds relies on the solvent used and the types of extraction (Skotti et al. 2014). This study has used an aqueous methanol (mixture of water and methanol at specific ratio) for extraction of free phenolic compounds. Phenolic compound in the free from can be easily extracted by using extraction solvents like aqueous methanol, ethanol and acetone (Amarowicz et al., 2008; Dabrowski & Sosulski, 1984; Troszynska & Ciska, 2002). Meanwhile the alkaline and acid hydrolyses were applied for releasing bound phenolic compounds. The extraction of free and bound phenolic acids can be done using

sequential alkaline extraction (Inglett et al. 2010). Acid hydrolysis assists to break the glycosidic bonds and solubilizes sugar leaving the ester bonds intact. Meanwhile, alkaline hydrolysis releases ester bond binding with phenolic acids to the cell wall (Monente et al. 2015). Till date, there is no specific method of extraction to obtain free phenolics in plants.

Antioxidant Activity of *C. nutans* Extracts using DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Assay

The antioxidant activities of *C. nutans* extract was determined by DPPH scavenging capacity assay. Wang et al. (2011) determined that DPPH is characterised as a stable free radical which delocalized electron or hydrogen by reduction process. When a solution of DPPH is mixed with substances that can donate a hydrogen atom, the change of violet to yellow colour occurs (Molyneux, 2004). Antioxidant or radical scavengers have the capability to accomplish this reaction. This method is widely used to determine radical scavenging capacity as it offers rapid assay as compared to other assay (Ebrahimzadeh et al. 2015). Redox properties in phenolic antioxidant might act as hydrogen donor, singlet oxygen quencher and reducing agents as well as metal chelating potential (Akinmoladun et al. 2010).

The antioxidant scavenging capacity can be ranked as follows: insoluble> soluble> free phenolic. This finding is parallel with a study by Ajila & Prasad Rao, (2013) which found significant amount of bound phenolics (soluble and insoluble) that contributed to the antioxidant properties in mango peel dietary fibre. The reducing power of bound phenolic (soluble and insoluble) in *Moringa oleifera* seed flour extract was significantly different as compared to free phenolic (Singh et al. 2013). This finding is consistent with a research done by Dvorakova et al. (2008) which found that insoluble bound phenolic acid was predominant in barley grains and malt and has significant antioxidant properties. Ongphimai et al. (2013) found that extraction of fruits in the form of insoluble phenolic had a higher percentage of radical inhibition in comparison to soluble bound phenolic content and ranked as followed: orange > mango > banana > guava.

Correlation between Total Phenolic Content and Antioxidant Radical Scavenging Capacity *C. nutans* Extracts

The correlation between antioxidant activities and TPC in different extracts can be analysed quantitatively. There is no significant difference found between TPC (free and insoluble bound phenolic) and antioxidant radical scavenging capacity. This statement is in agreement with Guo et al. (2011) who stated that the presence of non-phenolic compounds will contribute to the antioxidant activities. The positive correlation between free and insoluble bound phenolic with antioxidant radical scavenging capacity proved that the

high amount of TPC will contribute to high value of radical scavenging capacity percentage.

There was moderate negative correlation between soluble bound phenolic and antioxidant radical scavenging capacity. The results obtained show the soluble bound phenolic extract is high with TPC but low in antioxidant radical scavenging capacity. This study is in parallel with Ongphimai et al. (2013) which also found high phenolic content with low antioxidant capacity in insoluble phenolic of guava extract. Based on previous studies, there were various results obtained on the correlation between TPC and antioxidant activities; some researchers found strong correlation (Maizura et al. 2011), weak correlation (Meneses et al. (2013) and no direct correlation (Akinmoladun et al. 2010). Several studies found that, most spices and herbs contain phenolic compounds which attributed to the antioxidant properties (Maizura et al. 2011: Singh et al. 2013). Hence, it can be concluded that insoluble bound phenolic in *C. nutans* plant extract are able to exhibit radical scavenging capacity.

CONCLUSION

The total phenolic content and antioxidant activity of *C. nutans* extract in three different form of phenolic (free, soluble bound and insoluble bound phenolic) were determined. Different forms of phenolic showed different amount of total phenolic content and antioxidant activity. Insoluble bound phenolic demonstrated the highest amount of total phenolic as well as their antioxidant activity compared to free and soluble bound phenolics. According to Pearson test there was a strong positive correlation between TPC and DPPH radical scavenging capacity. The greater the amount of TPC in *C. nutans* extracts, the greater the ability of extract to scavenge free radical. In conclusion, the different forms of phenolic extract affect the TPC and antioxidant activities in *C. nutans* extract.

Future analysis of *C. nutans* should be done to improve the findings of bioactive compounds contained in this plant. The optimization of extraction parameter (temperature, time and types of solvent) should be emphasized to increase yield of phenolic acid content. Besides, determination and identification of specific compounds in *C. nutans* extract which possibly contributed to antioxidant properties can be done by using high performance liquid chromatography (HPLC).

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