TOTAL PHENOLIC CONTENTS AND FREE RADICAL SCAVENGING CAPACITY OF *CLITORIA TERNATEA* (BUNGA TELANG) EXTRACT

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ABSTRACT

Introduction: Most parts of *Clitoria ternatea* have been investigated for their medicinal purposes such as antimicrobial, antioxidant, anti-diarrheal, anti-diabetic, treatment for skin disease, sore throat, and memory enhancement. The present study aimed to investigate the total phenolic contents in free and bound forms (soluble and insoluble bound) and their radical antioxidant scavenging capacity. **Methods:** Free phenolic extract was acquired through direct methanol extraction while acidic and alkaline hydrolyses were adopted to release the bound phenolic. The total phenolic content (TPC)s were determined using Folin-Ciocalteu assay and expressed in gallic acid equivalents (GAE). The fractions were evaluated for their antioxidant capacity using DPPH assay. **Results:** The highest phenolic content was found in insoluble bound extract followed by soluble bound and free extracts (1.87 ± 0.05, 1.63 ± 0.05, 1.05 ± 0.11 mg GAE/ g DW, respectively). At the highest concentration of 50 mg/ml, soluble bound extract demonstrated the highest radical scavenging capacity compared to insoluble bound and free extracts (84.24 ± 1.96 %, 83.49 ± 1.03 %, and 63.37 ± 5.33 %). **Conclusions:** The insignificant differences between all the extracts and antioxidant inhibition in this present study suggested that different forms of extracts did not influence the radical scavenging capacity.

KEYWORDS: *Clitoria ternatea*, TPC, antioxidant, DPPH assay

INTRODUCTION

Phenolic compounds are a diverse group of phytochemicals which comprises of many different families of aromatic secondary metabolites of plants with more than 8000 structures currently discovered (Dai & Mumper, 2010). Polyphenols can be divided into three classes which are phenolic acids, flavonoids, and tannin. Flavonoids are one of the important phenolic compounds mentioned by Pokorny et al., (2001) to be very effective antioxidants which include flavones, flavonols, isoflavones, flavonones and chalcones while phenolic acids constitute of hydroxybenzoic and hydroxycinnamic acids. The differences in the number and location of hydroxyl groups on the aromatic ring contribute to the phenolic acids variation (Pereira et al., 2009). Ferulic, caffeic, *p*-coumaric and sinapic acids are the derivatives of hydroxycinnamic

acid while the derivatives of hydroxybenzoic acid are gallic, vanillic, syringic and protocatechuic acids (Khoddami et al., 2013). Tannins, the phenolic compounds that are capable to form oxidative linkage to other plant molecules, can be classified into two groups which are hydrolysable tannins and condensed tannins (Khoddami et al., 2013). On the other hand, majority of the soluble phenolic acids were localized and trapped in the vacuole of plant cells. Shahidi and Yeo (2016) concluded that most of the soluble and insoluble-bound phenolic acids were localised in the intracellular sites of plant cells. The most common chemical methods used to extract the insoluble-bound phenolics from cell wall matrices were mentioned by Shahidi and Yoe (2016) which were by acid and alkaline hydrolysis. Several medicinal plants that have potential to be used as alternatives to prescribed allopathic drugs currently available to treat various disorders and ailments are finding their way into the world market (Huang et al., 2005a). One of the most scientifically investigated-plants for its therapeutic values is *Clitoria ternatea*. It is commonly known as butterfly pea, a species that belong to the family Fabaceae. It is native of India and widely cultivated in the Caribbean area, Central America, Africa and Southeast Asia. According to Mukherjee et al., (2008) who investigated this plant in detail, the extract of *C. ternatea* exhibited a wide range of pharmacological and biological activities including anti-inflammatory, anti- diabetic, anti-microbial and antioxidant activities. It was reported that various chemical constituents are present in the roots, leaves, flowers and seeds of *C. ternatea* contains major phytochemicals like phenols, flavonoids, alkaloids, steroids and glycosides (Manalisha & Chandra, 2011). Although there has been much research regarding the effects of antioxidants exerted by phenolic compounds of *C. ternatea* on the human body, the investigation of antioxidant scavenging capacity in all forms of phenolic acids extracts of this plant and their relationship is rarely reported. Therefore, this research focuses on the determination of total phenolic compound in *C. ternatea*, the antioxidant activities in its respective form of free and bound extracts.

METHODOLOGY

Sample Collection and Preparation

The flowers of *Clitoria ternatea* were obtained from Terengganu and Kuantan, Pahang. The samples were pooled together and washed thoroughly with tap water before being cut into small pieces. They were put in a beaker and stored at -80 °C freezer for a day. The sample was freeze-dried for a week prior to grinding into powder by using mechanical blender. The powdered samples were stored in a dryer for the use of analysis.

Free Phenolic Extraction

The extractions of phenolic compounds from dried samples were performed according to Singh et al. ,(2013) method with slight modifications. About 1 gram of dried sample was extracted with 20 ml of 80% methanol. The mixture was homogenized using a vortex mixer for 5 minutes prior to sonication with Starsonic 60 sonicator (34 kHz, 175 W) for 1 hour at 30°C. The supernatant was then collected into a new falcon tube and the residues were kept at -80 °C for further analysis. The extraction operation was triplicated. The new supernatant produced upon the next sonication was pooled and filtered using Whatman filter paper No. 1. The extracts were subjected to desolventisation by using rotary evaporator (337 mbar, 60 °C) until semisolid residue was obtained. The semisolid residue was extracted 3 times with 10 ml diethyl ether and the organic phase was collected. The left-over semisolid was kept for further usage. The collected organic phase was evaporated to dryness using rotary evaporator (850 mbar, 60 °C). The dried extract was dissolved in 10 ml absolute methanol and being stored at 4 °C for further analysis as free phenolic extract.

Soluble Bound Extraction

The left-over semisolid residue obtained from the extraction with diethyl ether in free phenolic extract process was acidified with 6 N HCl to pH 2 after being hydrolysed with 20 ml of 4 M NaOH for 2 hours at room temperature. The sample was extracted 3 times with 10 ml diethyl ether and the organic phase was collected. The collected organic phase was then evaporated to dryness under vacuum (850 mbar, 60 °C). About 10 ml of absolute methanol was added to dissolve the residue before being stored at 4 °C for further analysis of soluble bound phenolic.

Insoluble Bound Extraction

The residue obtained from sonication in free phenolic extraction procedure was hydrolysed with 10 ml of 4 M NaOH at room temperature for 1 hour. The 6 N HCl was used to acidify the sample to pH 2 before subjected to centrifugation (4300 rpm, 10 min). The supernatant was collected and extracted with 15 ml of hexane to remove all forms of lipid contaminants. The sample was undergone evaporation to remove hexane using rotary evaporator (355 mbar, 69 °C). It was later extracted 3 times with 10 ml diethyl ether and the organic phase was collected. The collected organic phase was then evaporated to dryness under vacuum (850 mbar, 60 °C). About 10 ml of absolute methanol was added to dissolve the dried residue before being stored at 4 °C for further analysis of insoluble bound phenolic.

Folin-Ciocalteu's Assay

The assay was carried out based on the method done by Ismail et al., (2010) with some minor changes. About 100 µl of Folin-Ciocalteu reagent (10% v/v) was added to 6 µl of extracts of 30 mg/ml and the mixture was left for 5 minutes to react prior to the addition of 80 µl of sodium carbonate (5 % w/v). The absorbance at 760 nm was determined using microplate reader TECAN Infinite® M200 PRO after the mixture was incubated in the dark at room temperature for 1 hour with absolute methanol as a blank. All determinations were taken in triplicate and their averages were calculated. A standard curve was plotted and TPC of the extracts were expressed in mg gallic acid equivalent (GAE) per gram of dried-weight (DW) of sample. The total phenolic content was calculated by the following formula:

$$TPC\ \left(\%\frac{mg}{ml}\right) = \frac{+concentration\ of\ gallic\ acid\ \left(\%\frac{mg}{ml}\right) \times volume\ of\ samples\ (ml) =}{mass\ of\ the\ extract\ (g)}$$

DPPH Assay

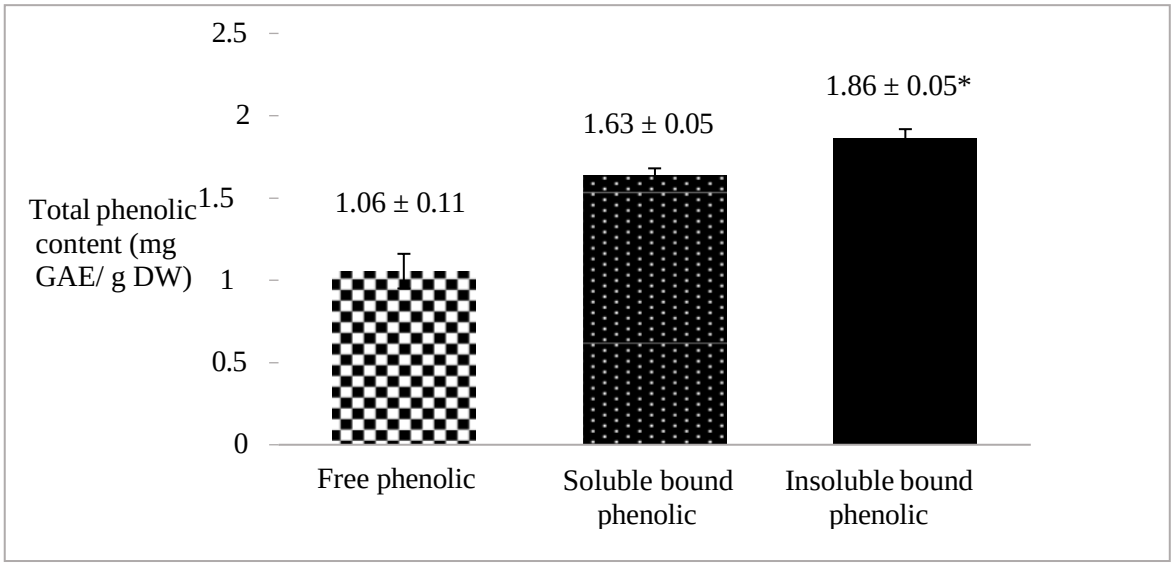
The antioxidant activities of the extracts were assessed using the method described by Kamkaen and Wilkinson (2009) with some modifications. The DPPH solution (0.004%) of was prepared by diluting 0.004g of DPPH powdered in 100 ml absolute methanol. Approximately 100 µl of the prepared DPPH solution was added to 100 µl extracts with 5 different concentrations produced through dilution with using absolute methanol according to the factors of 1:1, 1:5, 1:10, 1:15, and 1:20. The absorbance at 517 nm was determined using microplate reader TECAN Infinite® M200 PRO after 30 minutes incubation in the dark at room temperature. A mixture of 100 µl absolute methanol and 100 µl DPPH solution was functioned as positive control while absolute methanol acted as a blank. The DPPH scavenging activity was calculated as follows:

$$I(\%) = \frac{(A\ control - A\ sample)}{A\ control} \times 100$$

RESULTS

Total Phenolic Content of *C. ternatea*

The gallic acid standard curve was constructed by plotting down the absorbance at 760 nm against gallic acid concentration ranged from 0.01 mg/ml to 0.18 mg/ml. The total phenolic content in free, soluble, and insoluble bound extracts were calculated from the regression equation ($y = 2.1915x$; $R^2 = 0.9988$) and expressed in milligram of gallic acid per gram of dried weight (mg GAE/g DW) of sample. The Figure 1 describes the amount of total phenolic content in each extracts. The insoluble bound extract possessed the highest total phenolic content and followed by soluble bound and free extracts with 1.86 ± 0.10, 1.63 ± 0.05, and 1.06 ± 0.05 GAE/g DW, respectively ($p<0.05$). Significant differences between total phenolic content of insoluble bound and free extracts as well as soluble bound and free extracts were revealed when analysed using ANOVA ($p<0.05$).



Each value is expressed as mean of duplicate ± SD. * represent a significant different at the level of $p<0.05$

Figure 1 Total phenolic content of free, soluble bound and insoluble bound phenolic

Antioxidant Activity of *C. ternatea* using DPPH Scavenging Activity Assay

The gallic acid standard curve with R^2 of 0.9935 was calibrated using 5 different concentrations ranging from 0.0977 mg/L to 1.5625 mg/L. The radical scavenging capacity of the extracts was shown in Figure 2 to be concentration-dependent pattern. Generally, the antioxidant capacity of insoluble bound of *C. ternatea* extract was more vigorous at concentration below 10 mg/ml, after which the reaction gradually decelerated towards the highest concentration. At concentration of 10 mg/ml, the highest radical scavenging capacity was shown to be in insoluble phenolic extract with value of 47.7 ± 2.7 % compared to free and soluble bound phenolic extract of 44.78 ± 0.05 % and 39.27 ± 4.24 %, respectively.

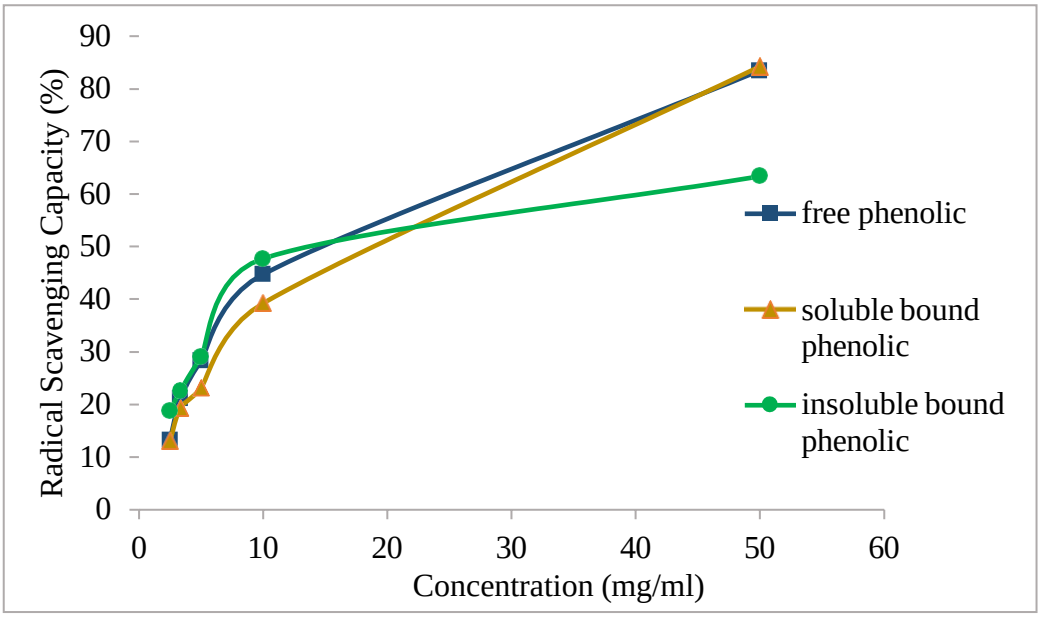


Figure 2 Scavenging capacities of three extracts on DDPH radicals

However, the trend started to change after that concentration. The soluble bound extract exhibited the highest percentage of radical scavenging capacity at the highest concentration of 50 mg/ml (84.24 ± 1.96 %) compared to free extract (83.49 ± 1.03 %) and insoluble bound extract (63.37 ± 5.33 %). The Table 1 shows the IC_{50} values for all extracts. There was an inversely proportional relationship between value of IC_{50} and radical scavenging capacity. The lowest value indicated the highest antioxidant radical scavenging capacity. The degree of DPPH reduction determines the radical scavenging capacity of particular extracts and their efficiencies as antioxidants also can be expressed in terms of IC_{50}. The value of IC_{50} represents the amount of antioxidants contained in extracts which needed to reduce 50% of free radical DPPH, calculated from the constructed graph of radical scavenging capacity against the concentration for each extracts. There were no significant differences between those extracts of *C. ternatea* and antioxidant inhibition when analysed using ANOVA, indicating that radical scavenging capacity and inhibition concentration were not influenced by the different forms of phenolic. The IC_{50} values for free, soluble and insoluble phenolic extracts are 23.10 ± 0.23, 24.32 ± 1.95 and 31.07 ±5.41 mg/L, respectively). Multiple comparisons calculated using post-hoc Tukey HSD (Honestly Significant Difference) also showed an insignificant difference between all three forms of *C. ternatea* extracts. To summarize, the different forms of extracts gave no effect to the radical scavenging capacity.

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

Type of extracts	**Average value of IC_{50} (mg/L) ±SD**
Free phenolic	23.10 ± 0.23
Soluble bound phenolic	24.32 ±1.95
Insoluble bound phenolic	31.07 ±5.41

DISCUSSION

Total Phenolic Content of *C. ternatea*

Total phenolic content of *C. ternatea* flower extract was quantified using Folin-Ciocalteu colorimetric method, involving the transfer of single electron from phenol to the complexed Mo(VI) contained in the reagent. It is widely used due to its simple procedure despites its inaccuracy in measuring the total phenolic content. As stated by Prior et al., (2005), many interfering compounds might react with the reagent, thus render it unspecific. Different phenolic compounds also had responded in different ways to this reagent, depending on the number of phenolic groups they possessed (Tawaha et al., 2007). Siatka and Kašparová (2010) explained that total phenolic content can also be underestimated as different type of phenolics reacted with Folin-Ciocalteu reagent in different ways. This finding (Figure 1) was in agreement to the result from the previous research which reported that the bound phenolic acids content was significantly higher than the other fractions (Madhujith & Shahidi, 2009). A consistent result also was obtained by Kaisoon et al., (2011) who investigated the total phenolic content in both free and bound phenolic forms from 12 available Thai edible flowers. The authors mentioned that *B. hybrida* and *N. nucifera* had a higher phenolic content in bound phenolic extract than in its free form. Free form of phenolic had occurred in plant tissues at a minimal quantity due to their toxicity level present in a free state and detoxified at least in part when bound (Giada, 2013). However, by totaling up the total phenolic content of all three different forms of phenolic, it still exhibited a comparable value to the previous research done by Rabeta and Nabil (2013) who found that the total phenolic content in *C. ternatea* flower extracted using methanol was about 61.7 GAE per gram flower. This could possibly due to the different sources of the flower and its maturity state since the samples in the present study were randomly collected from various places and time. According to Siddhuraju and Becker (2003), different value of total phenolic content had been quantified from *M. oleifera* leaf which obtained from three different agro climatic conditions of India, Nicaragua and Niger ranged from 2940 to 4250 mg GAE/100 g dry weight. A reduction in phenolic and anthocyanin were observed by Takahama *et* al., (1997) at the later stages of flower development.

Antioxidant Activity of *C. ternatea* using DPPH Scavenging Activity Assay

The capacity of the three different *C. ternatea* extracts in reducing the oxidants were determined spectrometrically at 517 nm based on their color changes during reduction process. The antioxidant concentration of extracts influenced the degree of color change. However, there was no universal method that was capable of taking into account all modes of antioxidant's action (Huang et al., 2005b; Prior et al., 1999). At the concentration of 10 mg/ml, the highest radical scavenging capacity was shown to be in insoluble phenolic extract as compared to free and soluble bound phenolic extract (Figure 2). This was in line with the previous study done by Liyana-Pathirana and Shahidi (2006) which demonstrated that the highest antioxidant potential was found in insoluble bound phenolic of wheat as compared to soluble ester and free forms of phenolic. The similar pattern of antioxidant capacity was also observed when studied with ten different types of barley grains (Dvořáková et al., 2008). However, the trend started to change after that concentration. The soluble bound extract exhibited the highest percentage of radical scavenging capacity at the highest concentration of 50 mg/ml as compared to free extract and insoluble bound extract. The trend shown by insoluble bound extract might be explained for their antioxidant compounds that had changed their redox properties to be pro- oxidants at the higher concentration. According to Kong et al., (2012), some antioxidants are able to act as pro-oxidants. The statement was supported by Procházková et al., (2011) who asserted that flavonoids could turn into pro-oxidants, depending on the number of hydroxyl groups in their structures, their concentration, and the presence of transition metal ions. Different affects can be exerted by antioxidants via different mechanisms. Besides being free radical scavengers (Ragaee et al., 2006), phenolic and other antioxidants found in cereals also can act as singlet quenchers, electron donors, as well as chelating of

pro-oxidant metals ion (Zielinski, 2002). Due to these multifunctional properties of antioxidants, it is necessary to perform multiple analysis which represent different action mechanisms for determination of antioxidant capacity of phenolic (Wong et al., 2006). The degree of DPPH reduction determines the radical scavenging capacity of particular extracts and their efficiencies as antioxidants also can be expressed in terms of IC_{50}. The value of IC_{50} represents the amount of antioxidants contained in extracts which needed to reduce 50% of free radical DPPH, calculated from the constructed graph of radical scavenging capacity against the concentration for each extracts. There were no significant differences between those extracts of *C. ternatea* and antioxidant inhibition when analysed using ANOVA, indicating that radical scavenging capacity and inhibition concentration were not influenced by different forms of phenolic. Different form of phenolic extracts might constitute of different types of phenolic compounds, which eventually contribute to the different total antioxidant capacity. According to Sengul et al., (2009), the different type and structure of phenolic compounds, antioxidant scavenging capacity also affected by the presence of other phytochemicals, for instance, tocopherol, pigments, and ascorbic acid. The synergistic effects between them might also alter the overall antioxidant capacity. To conclude, the present findings suggested that high antioxidant scavenging capacity demonstrated through DPPH assay might not necessarily be due to the phenolic compounds in the extract of *C. ternatea* flower.

CONCLUSION

The total phenolic contents were varied dependent upon the classes of phenolic either free, soluble bound or insoluble bound. Insoluble bound possessed the highest total phenolic content compared to soluble bound and free form of phenolic despites of no significant difference in the antioxidant radical scavenging capacity shown by those three extracts, indicated that radical scavenging capacity were not influenced by different forms of phenolic. In the future, optimization of parameters involved in the pre-treatment samples stage should be carried out in order to increase the yield of total phenolic content and further eliminates the interferences besides exploring more precise assays for phenolic. Further identification and characterization of the phenolic compounds possessed by *Clitoria ternatea* flower has to be undertaken using well-developed chromatography technique so that those enhancing and supressing the antioxidant activity in all forms of phenolic (free, soluble or insoluble bound) could be known.

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