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Total phenolic compounds and scavenging activity in *Clitoria ternatea* and *Vitex negundo* linn

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Abstract

This study was done to assess the total phenolic compounds (TPC) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity in the flowers and leaves of *Clitoria ternatea* and *Vitex negundo* Linn. by using methanol and water extraction. TPC were evaluated using Folin-Ciocalteau method. Methanol was more efficient in extracting phenolic compounds compared with water in measuring TPC. *Vitex negundo* Linn. contained higher amount of TPC compared to *Clitoria ternatea*. Besides that, leaves for both plants showed higher amount of TPC compared to the flowers. Methanol extracted *Vitex negundo* Linn. showed higher DPPH scavenging activity compared with *Clitoria ternatea*. In contrast, DPPH scavenging activity for water extracted *Clitoria ternatea* showed higher value in compare with water extracted *Vitex negundo* Linn.. The type of solvent used to extract the plant material and concentration of extracts used showed significance difference ($P < 0.05$) on the amount of DPPH scavenged by the plant extract. The presence of antioxidant activity in both leaves and flowers showed that *Clitoria ternatea* and *Vitex negundo* Linn. have the potential to be an alternative source of natural antioxidants. *In vivo* study is needed for successful commercialization and to benefit both the food and pharmaceutical industries.

Introduction

Lipid peroxidation is one of the factors that may affect appearance, flavour, and nutritional quality in many processed foods. The process is also important for the stability of processed food (Aruoma, 1999). In the process of lipid peroxidation, many radical species such as lipid alkoxyl radical, are generated and cause not only food deterioration but also DNA, cell, and tissue damage (Nakatani, 1994).

In response, natural or synthetic antioxidants have been widely used to delay or prevent lipid peroxidation process in a variety of food products. These naturally occurring compounds impart bright colour to fruits and vegetables and act as antioxidants in the body by scavenging harmful free radicals, which are implicated in most degenerative diseases (Kaur and Kapoor, 2001). During lipid peroxidation, antioxidants may act in various ways such as binding metal ions, scavenging free radicals, and decomposing peroxides (Moure *et a*l., 2001). Antioxidants also defend our immune system to fight against reactive oxygen species (ROS) especially in the process of ageing.

Plants and herbs have been an important contributor to the quality of human life for thousands of years. Some of them are well known medicinal herbs. Butterfly pea or blue pea (*Clitoria ternatea*) from family of Fabaceae is a vine with vivid blue flowers 1 to 2 inches long, having wavy-rimmed standard and white centre, which is rather common in gardens of Hawaii (Neal, 1968). *Clitoria ternatea* flower are commercially known as *Bunga telang* by the locals and are widely used as the food dyes in Nasi kerabu (the local dish in Kelantan, Malaysia) and a Baba and Nyonya kueh known as kueh tekan. The flowers are used in those products just for the purplish blue colour from its petals without knowing their health benefit.

Leggundi (*Vitex Negundo* Linn.) commonly known as ‘‘lemuni hitam’’ in Malaysia is from family of Verbenaceae. Verbenaceae family commonly has been known as having quadrangular branches (Neal, 1968). Recently, indigenous *Vitex negundo*’s

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leaves are used to make ''Nasi Lemuni'', a famous indigenous food in the northern area of Peninsular Malaysia. In addition, the young leaves of the plant are used as one of the traditional remedy for the postnatal women. An essential oil of *Vitex negundo* Linn. is found to be useful for outer layer of wounds and ulcers (Khokra *et al*., 2008).

The addition of antioxidants is required to preserve flavour and colour and to avoid vitamin destruction in processed food (Moure *et al*., 2001). Now, as then, recent increasing interest in natural antioxidant found in plant sources has become a trend, because people worldwide are focusing on using natural additives, not only in food, but also in cosmetics industry. As a whole, antioxidant has become such popular topic to be discussed mainly on their effect on preventing the risk of chronic disease in many types of cancer. As the global cancer incidence is increasing, researchers and health leaders around the world are scratching their heads and trying to understand the mechanisms to reduce the risk of this frightening disease. There has been a lot of interest in natural antioxidants since the possible side effects of synthetic antioxidant such as butylated hydroxyanisole (BHA) (Munday *et al*., 1998; Munday *et al*., 1999). Therefore, there have been numerous researches on crude extraction and bioactive compound of potential plants for natural and possibly economic and effective antioxidants to replace the synthetic ones. Plants are great alternatives in many local communities in Malaysia and Asia in general, used as health supplements and therapeutic (Khatijah and Mohamad Ruzi, 2006)

These plants have not received much attention as antioxidant sources compared to others plants in Malaysia due to the lack of information about total phenolic compounds (TPC) and scavenging activity of *Clitoria ternatea* and *Vitex negundo* Linn.. Therefore this study aimed to assess the TPC in the flowers and leaves of *Clitoria ternatea* and *Vitex negundo* Linn. by using two types of extraction, namely methanol and aqueous. This study was also undertaken to evaluate the antioxidant activity possessed by both plants, by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.

Materials and Methods

Plant material

Fresh flowers and leaves of both *Clitoria ternatea* and *Vitex negundo* Linn. were collected form Kg. Seronok, Bayan Lepas, Pulau Pinang, Malaysia. Fully bloomed flower of both *Clitoria ternatea* and *Vitex negundo* Linn. were collected for the study. As for *Clitoria ternatea* leaves, only those with good shape were selected, while for *Vitex negundo* Linn., the leaves chosen were basically the young shoots, that happened to have completely purple colour on the underneath part of the leaves.

Sample preparation

In brief, the collected flowers and leaves were separated from their stems and dried in a vacuum oven (Binder Vacuum Oven Model VD53) at 30°C for 12 hours. Dried raw materials were left in desiccators for 24 hours. All the dried flowers and leaves were ground using normal grinder and sieved. Plant powders were stored in an air-tight polyester container and stored at -20°C (Lab Freezer Protech, Model CR400) prior to the analysis.

Methanol extract of leaves and flowers

Based on the method proposed by Fu *et al*. (2009), 5 g of powder were added to 100 ml of 70% methanol. Mixture was kept in orbital shakers (Lab Companion, Model SI600R) overnight and then filtered using Whatman No. 1. Filtrate was vaporized in a rotary vacuum evaporator (Heildoph VV2200) by using a water bath (Büchl Waterbath Model B-480) at 45°C and vacuum pump to obtain dry extract.

Aqueous extract of leaves and flowers

Aqueous extraction of plant material was done based on the method proposed by Wong *et al*., (2006). Briefly, 0.5 g of plant powder was extracted using 25 ml of deionised water. The mixture was allowed to stand in dark area at room temperature for 1 hour, with occasional agitation. By using filter paper (Whatman No.1), the mixture was filtered and supernatant obtained was used for analysis without further treatment.

Total phenolic content determination

Total phenolics content in methanol extract of flowers and leaves of both plants were determined using the method proposed by Kaur *et al*. (2006) with slight modification. A total of 100 mg of dry extract was leached using 250 ml of methanol/water (60:0, v/v, 0.3% HCl) and then filtered using filter paper (Whatman No 1). For every 100 µl of filtrate, about 100 µl of 50% Folin-Ciocalteau reagent and 2 ml of 2.5% sodium carbonate were added. The mixture was mixed completely and allowed to stand for 2 hours. Then, the absorbance of the solution at 750 nm was measured. Quantification of TPC was done using standard curve of gallic acid as a standard phenolic compound (0.01-0.05 mg/ml), which was dissolved in methanol/water (60:40, v/v, 0.3% HCl) and expressed as mg gallic acid per gram of plant material. Sample measurements were done in

triplicates and the mean and standard deviations were calculated in each case.

For aqueous extracts, the TPC was determined based on the method by Wong *et al*., (2006). About 100 µl (20 µg/ml) of the extract was mixed with 2.5 ml Folin-Ciocalteau reagent (10 times dilution) and 2.5 ml of 2.5% sodium carbonate solution. The mixture was allowed to stand for 1 hour and the absorbance was measured at 725 nm. Total phenolics content was quantified using standard curve of gallic acid as a standard phenolic compound (0.2 to 1 mg/ml), which was dissolved in deionised water and expressed as mg gallic acid equivalent (GAE) per gram plant material.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity determination

Scavenging activity of extracts against DPPH radical was measured based on the method proposed by Gülcin *et al*. (2006) and by Fu *et al*. (2009). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added with 3 ml of respective extracts solution (aqueous or methanolic extract) at five different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using UV-VIS spectrophotometer (Shimadzu UV Visible Recording Spectrophotometer Model UV-160A). Lower absorbance of the mixture indicates higher free radical scavenging activity.

Positive control was prepared by mixing 2 ml of ascorbic acid (0.05mg/ml) and 1 ml of DPPH (0.04mg/ml), whereas negative control was prepared by mixing distilled water with 1 ml of DPPH. About 3 ml of the mixture (final concentration of 25, 50,100, 125 and 150 µg/ml) prepared from working solution was added to 1 ml of DPPH. The mixture was gently homogenized and left to stand at the room temperature for 30 min. Absorbance was read using spectrophotometer (Shimadzu UV Visible Recording Spectrophotometer Model UV-160A) at 517 nm.

Statistical analysis

All experiments were repeated three times. Results for total phenolic contents were reported as means ± SD. Significant differences for multiple comparisons were determined by one-way analysis of variance (ANOVA) followed by Duncan test with p value less than 0.05 which was considered as statistically significant by SPSS statistical package (ver.17.0).

Results and Discussion

Total phenolic compounds

The amount of TPC in plant extract was based on the absorbance of sample and Folin-Ciocalteau reagent mixture at 725 nm. The amount of total phenolic compounds that present in each of the samples was reported as mg GAE per gram sample. Since Folin-Ciocalteau assay measures all the phenolics, the choice of gallic acid as standard is based on the availability of a stable and pure substance, and gallic acid is both, and it is less expensive than other options. Two standard curves were plotted to quantify the phenolic compound in samples as mg GAE per gram samples, one for methanol extract and another one for water extraction. The total amount of TPC in each sample was represented as mean mg GAE per gram sample (Table 1).

Generally, the amount of TPC exhibited by methanol plant extract (61.7 – 93.7 mg GAE per gram plant parts) was higher compared with aqueous plant extract (18.5 – 25.8 mg GAE per gram plant parts). Based on the results obtained, phenolic compounds in both plants are more soluble in methanol compared with water. Phenolic compounds and their derivatives are secondary metabolites in many plants. They are safe to be consumed by human and animal (Chew *et al*., 2009). Basically, the chemical forms of natural phenolic acids are distinguishable by hydroxycinnamic and hydroxybenzoic acids structures (Qiu *et al*., 2010).

The amount of TPC for methanol extract in *Clitoria ternatea* were 61.7 mg GAE per gram flower and 64.8 mg GAE per gram leaves while for water extract, 20.7 mg GAE per gram flower and 18.5 mg GAE per gram leaves. Comparing this result with that of *Vitex negundo* Linn., the amount of TPC from methanol extract of flowers and leaves were 83.3 mg GAE per gram and 93.7 mg GAE per gram, respectively. Lower TPC was obtained from water extract of both samples, which were 18.8 mg GAE per gram and 25.8 mg GAE per gram, respectively. Both amount were lower compared to total phenolic compounds found in *C. siamea* flower with 257 mg GAE per gram sample (Kaur *et al*., 2006) but higher compared to TPC found in pineapple (34.7 mg GAE per 100 gram) and guava (153 mg GAE 100 gram) as reported by Alothman *et al*. (2009).

This study found that *Vitex negundo* Linn. contains higher amount of TPC compared to *Clitoria ternatea*. For *Vitex negundo* Linn., TPC in methanol and water extract ranged from 83.3 to 97.3 mg GAE per gram sample and 18.8 to 25.8 mg GAE per gram, respectively. While TPC obtained were 61.7 – 64.8

Table 1. Comparison between total phenolic compound in *Clitoria Ternatea* and *Vitex Negundo* Linn. Of Methanol and Water Extract

Plant species	Parts used	mg GAE per g samples	
		Water extraction	Methanolic extraction
Clitoria ternatea	Flowers	20.7 ± 0.1[b]	61.7 ± 0.2[a]
	Leaves	18.5 ± 0.4[a]	64.8 ± 0.1[b]
Vitex negundo Linn.	Flowers	18.8 ± 0.1[a]	83.3 ± 0.1[c]
	Leaves	25.8 ± 0.2[c]	93.7 ± 0.4[d]

* Means with different superscript in any column are significantly different at $p < 0.05$, according to Duncan's Multiple-Range Test (n = 3)

mg GAE per gram sample and 18.5- 20.7 mg GAE per gram samples for methanol and water extraction of *Clitoria ternatea*, respectively.

Phenolic compounds are water soluble natural antioxidants which normally an aromatic ring bearing one or more hydroxyl substituent. They are usually located in the vacuole of the plant cells and may combine with sugar, to be glycosides (Waterhouse, 2002). When comparing TPC based on the parts of plant used, *Clitoria ternatea* flower exhibited higher TPC content compared with the leaves part in water extract sample. This result obtained were supported by statements by Terahara *et al*., (1998) and Sethiya *et al*., (2009) even though pigments present in *Clitoria ternatea* are low but it has high stability in water. Because of its high stability in aqueous solution, the flower pigment is used in South-East Asia as food colorant (Terahara *et al*., 1998; Mukherjee *et al*., 2008) had also reported the wide use of the blue flowers of the plant due to its stability. Going further, according to Sethiya *et al*., (2009) the extractive value (w/w, %) of *Clitoria ternatea* is higher in water (18.21%) compared to its extractive value in alcohol (16.14%). However, in methanol extracted sample, *Clitoria ternatea* leaves part contained higher TPC. This situation may happen due to the solubility nature of plant phenolics that has been enhanced by the organic solvent (methanol), which facilitates solubilisation through penetration in plant cell structure (Moure *et al*., 2001).

Folin-Ciocalteau assay only gave crude estimate of the total phenolic compound present in an extract. It does not measure specific polyphenols, but many interfering compounds may react with the reagent, thus giving elevated apparent phenolics concentrations (Prior *et al*., 2005). Moreover, Tawaha *et al*., (2007), stated that various phenolic compounds will respond differently to the reagent, depending on the number of phenolic groups they have and total phenolic does not incorporate necessarily all antioxidant that may be present in the fraction or extract.

Several compounds may contribute to the amount of TPC in the plant extract. According to Naczk and Shahidi (2004), those compounds are mainly phenolic acid, flavonoids, and anthocyanins. Commonly, flavonoids will be extracted from plant materials together with methanol, ethanol, or water that was used as extractor solvent.

Anthocyanin may contribute to the amount of TPC obtained from the plant samples as it is one of the important group of water-soluble pigments in plants (Clifford, 2000). This pigment is very important in both Clitoria ternatea flower and *Vitex negundo* Linn. leaves as their colour were blue and purple. Acidic methanol has been used to leach the dry methanol extract. Naczk and Shahidi (2004), stated that anthocyanin is usually extracted from plant material by using acidified organic solvent especially methanol. Acidified organic solvent used will destroy cell membranes, simultaneously dissolves anthocyanin thus stabilizing them.

DPPH free radical-scavenging activity

Determination of DPPH scavenging activity in both plants, methanol and aqueous extract, was done in triplicates. DPPH is a compound that consists of a nitrogen free radical which is easily destroyed by a free radical scavenger (Chew *et al*., 2009). Scavenging activity of the mixture was measured using UV-Vis at 517 nm after being incubated for 30 min in a dark place. Five different concentrations of samples extracts were tested to identify the significance of extract concentration on the scavenging activity. Water and DPPH mixture was used as negative control while ascorbic acid was used as positive control (Fu *et al*., 2009).

The amount of scavenging activity in each plant extract of both extractions is shown in Table 2. From the table, it can be observed that, percentage of DPPH scavenging activity for all plant extract increased with the concentration of plant extract used. This statement is true for all methanol plant extract with concentration; 25, 50, 100 and 125 µg/ml. However, at 150 µg/ml extract concentration, the scavenging activity of the extract decreased (like in Clitoria ternatea flowers, *Vitex negundo* Linn. flowers and leaves) or maintained at the same amount like for the 125 µg/ml extracts.

It can be seen that *Vitex negundo* Linn. extracts exhibited more scavenging activity towards DPPH radicals compared to *Clitoria ternatea* extract in methanol extracted sample solution. A study done by Tiwari and Tripathi (2006) has shown that all fraction of *Vitex negundo* Linn. have potent scavenging activity for ABTS radical cation in a dependent manner. They explained that this situation may be attributed to the existence of polyphenols and flavonols in *Vitex negundo* Linn.

Table 2. DPPH scavenging activity in plant extracts (methanol and water extracts)

Extraction solvent	Sample and parts	% scavenging activity per sample extract concentration (ug/ml)				
		Sample Concentration (µg/ml)				
		25	50	100	125	150
Methanol	*Clitoria ternatea* flower	32.67 ± 1.155^{a}	353.33 ± 3.055^{b}	411.33 ± 1.155^{d}	422.67 ± 3.055^{e}	401.33 ± 2.309^{c}
	Clitoria ternatea leaves	64.67 ± 1.155^{a}	264.00 ± 4.00^{b}	408.67 ± 4.163^{c}	472.00 ± 4.00^{d}	472.00 ± 4.00^{e}
	Vitex negundo Linn. flower	399.33 ± 3.055^{a}	442.00 ± 2.000^{b}	471.33 ± 3.055^{d}	491.33 ± 1.155^{e}	464.00 ± 4.000^{c}
	Vitex negundo Linn. leaves	453.33 ± 2.309^{d}	402.67 ± 2.309^{a}	514.67 ± 4.619^{e}	437.33 ± 4.619^{c}	406.67 ± 2.309^{b}
Water	*Clitoria ternatea* flower	390.67 ± 2.309^{a}	401.33 ± 3.055^{b}	449.33 ± 2.309^{c}	490.67 ± 4.619^{d}	506.67 ± 2.309^{e}
	Clitoria ternatea leaves	2.67 ± 1.155^{a}	8.67 ± 1.155^{b}	103.33 ± 1.155^{d}	102.67 ± 4.619^{c}	130.67 ± 4.619^{e}
	Vitex negundo Linn. flower	145.33 ± 2.309^{a}	273.33 ± 2.309^{b}	410.67 ± 4.619^{e}	398.67 ± 6.110^{d}	394.67 ± 1.155^{c}
	Vitex negundo Linn. leaves	184.00 ± 2.00^{a}	284.67 ± 1.155^{b}	391.33 ± 1.155^{c}	404.67 ±3.055^{e}	392.67 ± 3.055^{d}

* Means with different superscript in any column are significantly different at $p < 0.05$, according to Duncan's Multiple-Range Test (n = 3)

In contrast, DPPH scavenging activity in water extracted sample greatly deviates between both samples. *Clitoria ternatea* flower exhibited very high scavenging activity by the antioxidant compared with the scavenging activity expressed by both parts of *Vitex negundo* Linn. and *Clitoria ternatea* leaves. This complies with the result of total phenolic compounds that has been discovered before. For total phenolic compounds in water extracted *Clitoria ternatea* flower, high amount of total phenolic compounds has been found. Thus, it can be concluded that scavenging activity expressed by *Clitoria ternatea* flower was affected by the amount of total phenolic compound. In a study by Chew *et al*., (2009), significantly higher phenolic content and strong free radical scavenging activity were found in *B. Kockiana* flower compared with the leaf. This was due to the role of anthocyanin as antioxidant.

Generally, it is well known that plant phenolics are highly effective free radical scavengers and antioxidants. The research presented by Maisuthisakul *et al*., (2007) has reported that the phenolics compounds and its derivatives such as phenolic acids and tannins were strongly correlated with antioxidants. In this study, all samples did followed the trend but the only sample that totally exhibit this trend was water extracted *Clitoria ternatea* flower; which has been shown to have both high TPC and scavenging activity towards DPPH radical. The purple colour in both plants could be due to anthocyanin compounds. Study by Alothman *et al*., (2009) had shown that there was a significant correlation between total phenolics and antioxidant capacity of fruits extracts, namely honey pineapple, pisang mas, and guava and this correlation confirms that the phenolic compounds are the main micro constituents contributing to the antioxidant activities of these fruits.

Conclusion

It can be concluded that methanol is a more efficient solvent in extracting phenolics compound compared with water. Total phenolics compound found in methanol extracted sample was between 61.7 to 64.8 mg GAE per gram samples for *Clitoria ternatea* while 83.3 to 93.7 for *Vitex negundo* Linn.. As for water extracted sample, range of TPC obtained was between 18.5 to 20.7 mg GAE per gram samples for *Clitoria ternatea* and 18.8 to 25.8 for *Vitex negundo* Linn. *Vitex negundo* Linn. contained higher amount of total phenolics compound compared to *Clitoria ternatea* with leaves of both plants showed higher amount of phenolics compound compared with the flower part. As for the DPPH scavenging activity, type of solvent used to extract the plant material and concentration of extracts used produced significance difference on the amount of DPPH scavenged by the plant extract. Both *Clitoria ternatea* and V*itex negundo* Linn. are readily available and can be a source of natural antioxidants. Further studies are needed to evaluate the antioxidant activities in Nasi

Kerabu and Nasi Lemuni after mixed with both plants.

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