TOTAL PHENOLICS CONTENTS AND FREE RADICAL SCAVENGING CAPACITY OF *PIPER SARMENTOSUM* (PUCUK KADUK) EXTRACT

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ABSTRACT

Introduction: *Piper sarmentosum* is one of the herbaceous plants that has been used as natural antioxidant to source to treat diseases. This study was conducted to determine the total phenolic contents (TPC) and free radical scavenging capacity in free and bound (soluble and insoluble) of *P. sarmentosum.* **Methods:** Free phenolic extract was acquired through direct methanol extraction while acidic and alkaline hydrolyses were adopted to release the bound phenolic acids. The TPC was determined by using Folin-Ciocalteu assay and is expressed as Gallic Acid equivalent (GAE) in miligrams per gram of extracts. The antioxidant scavenging capacity was determined by using DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay. **Results:** Insoluble bound phenolic extract of *P. sarmentosum* showed the highest TPC value (1.54 ± 0.04 mg GAE/g DW) followed by soluble phenolic extract and free extract (1.13 ± 0.10 and 0.57 ± 0.06 mg GAE/g DW, respectively). The soluble phenolic fraction has expressed the highest free radical scavenging capacity (76.57± 4.12%) followed by insoluble (69.79± 2.33 %) and free extracts (58.15± 4.44 %). The IC_{50} values for free, soluble and insoluble bound phenolic were 24.05 ± 3.81, 16.17 ± 1.84 and 18.49 ± 1.92 mg/ml, respectively. **Conclusions:** The significant differences between all the extracts and antioxidant inhibition in this present study suggested that different forms (free and bound) of extracts did influence the radical scavenging capacity as a whole.

KEYWORDS: TPC, radical scavenging, DPPH assay, *Piper sarmentosum*

INTRODUCTION

Free radicals or reactive oxygen species (ROS) are produced continuously in the body as a result of endogenous metabolism or by the exposure to exogenous pollutants. According to Ugusman *et al*. (2012), higher concentration of free radicals causes destruction of the cellular components, leading to cell death. There is endogenous production of antioxidant system that can scavenge the free radicals such as catalase and superoxide dismutase (SOD), but these are inadequate to completely remove the free radical and maintain a healthy balance. Commercialized synthetic antioxidants such as butylated

hydroxytoluene (BHT) and hydroxyanisole (BHA) are reported to lead to carcinogenesis and produced other negative side effects. Therefore, natural antioxidants are needed to be incorporated in the diet such as edible raw plants, spices and herbs in order to stabilize these excess free radicals.

The herbal remedies were used to treat many infectious diseases in the past of (Zaidan *et al*., 2005) and are still in use for many disease conditions. The use of raw plants to treat human illnesses is due the reason that it contains low toxicity good therapeutic results (Li *et al*., 2008). Approximately 2000 plant species in Malaysia are reported to have medicinal properties (Abu Bakar *et al*., 2011). Malaysian consumes raw plants as their traditional diet which they believe to have health-promoting properties (Chan *et al*., 2014).

There are a lot of edible raw plants in Malaysia such as *Anacardium occidentale* (cashew), *Morinda elliptica* (noni or cheese fruit), *Piper betle* (betel), *Piper sarmentosum* (wild pepper), *Zingiber officinale* (ginger), and others. *P. sarmentosum* is one of the herbaceous plant that is widely found in tropical and subtropical regions of which leaves and roots are used to treat toothache, fungoid dermatitis, asthma and pleurisy (Hafizah et al., 2010). Hafizah et al. (2010) reports that Malaysians commonly consume it raw for the treatment of common diseases such as hypertension and diabetes mellitus. The aqueous extract of *P. sarmentosum* also has been reported to improve endothelial function by promoting nitric oxide production in human umbilical veins (Ugusman et al. (2012) and Hafizah et al. 2010). There are a few studies which show the presence of antioxidant activity in the *P. sarmentosum* (Li et al., 2008; Hussain et al., 2009).

Phenolic are broadly circulated in the plant kingdom and are the most abundant secondary metabolites of plant (Dai & Mumper, 2010). This compound has a great potential to possess antioxidant properties. According to Chan et al. (2016), compounds that have phenols in their structure are commonly recognized as antioxidants because they can serve as free radical scavenger. Natural antioxidant has a lot of beneficial effects on health and can be consumed from the raw plant sources (Kusuma et al., 2014). Previous studies have focused on free form of phenolic compound only (Habila et al., 2010; Kim et al., 2010). Therefore, this research will focus on the determination of total phenolic contents in both free and bound extract of *P. sarmentosum* and to identify its relationship with antioxidant scavenging capacity.

METHODS

Collection and Preparation of *Piper sarmentosum*

P. sarmentosum or wild pepper/kadok leaves were collected from Bukit Setongkol, Kuantan, Pahang. The leave samples were washed and kept in -80^{O}C freezer before the freeze drying process for a week. The freeze-dried plants were ground into fine powder by using electronic blender and stored in the freezer (-20 ^{O}C) until further analysis.

Free Phenolic Extraction

The free phenolic extraction method was followed the method as described by Singh *et al*. (2013). One gram of sample was mixed with 20 ml of 80% methanol. After vortex, the samples were mixed by using sonicator for 1 hour. The residue was kept as bound insoluble phenolic for further analysis. Meanwhile, the supernatant was filtered by using Whatman filter paper No.1. These steps were repeated twice. After filtered, the supernatant was evaporated by using rotary evaporator until semi solid residue was

obtained. Then, the residue was again extracted with diethyl ether for three times. Two layers were obtained which are aqueous phase and organic phase. The aqueous phase was kept as bound soluble phenolic. Meanwhile, organic phase was collected and then evaporated to dryness. The dried residue obtained was mixed with 10 ml methanol and kept under -80^{O}C as free phenolic for further analysis.

Bound Soluble Phenolic Extraction

The bound soluble phenolic extraction method was followed the method as described by described by Singh *et al*. (2013). The bound soluble phenolic acid was mixed with 20 ml of 4M sodium hydroxide and stored for two hours at room temperature. The sample was then acidified to pH 2 by adding 6M hydrochloric acid. Then, the sample was extracted with diethyl ether for three times. The organic phase (contain bound soluble phenolic contents) was collected and evaporated to dryness by using rotary evaporator. The dried residue was then dissolved with 10 ml methanol and kept under -80^{o}C for further analysis.

Bound Insoluble Phenolic Extraction

The bound insoluble phenolic extraction method was followed a method as described by Abdel-Aal *et al*. (2012). The residue was mixed with 10 ml of 4M sodium hydroxide and store for 1 hour at room temperature. The pH of the sample was adjusted to pH 2 by adding 2M hydrochloric acid. Then, the sample was centrifuged for 10 minutes at 4380 rpm. The supernatant was collected and extracted with 15 ml hexane. The aqueous layer was then extracted three times with diethyl ether. The organic phase was collected and evaporated to dryness. The dried residue was dissolved in 10 ml methanol and kept under -80^{o}C for further analysis as insoluble phenolic.

Folin-Ciocalteu's Assay

Folin-Ciocalteu's Assay was followed a method as described by Singh *et al*. (2013). The assay was used to determine total phenolic content in the plant sample and the gallic acid was used as a standard. 0.2 mg/ml of gallic acid stock was prepared by adding 10 mg of gallic acid powder into 50 ml of methanol. Ten different concentration of standard were prepared in the range from 0.02 - 0.2 mg/ml. Six microliter samples/blank were transferred into the microplate. Then, 100 µl of Folin-Ciocalteu reagent was mixed with the sample/blank. After 5 minutes, 80 µl of sodium carbonate was added and gently mixed. The mixtures were then incubated for 1 hour at room temperature. The absorbance was measured at 760 nm by using spectrophotometer. The analyses were done in triplicate and the results were expressed as miligrams Gallic Acid Equivalents per gram, of extract weight (mg GAE/ g extract).

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

The antioxidant activities of the extracts were assessed using the method described by Kamkaen and Wilkinson (2009). The standard stock solution of 25 mg/L was prepared by dissolving 2.5 mg of gallic acid powder in 100 ml of absolute methanol. The stock solution was diluted with absolute methanol via serial dilution to produce 5 different concentrations (0.0977, 0.1953, 0.3906, 0.7813, and 1.5625 mg/L). 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 µlextracts 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 aspositive control while absolute methanol acted as a blank.

Statistical Analysis

All experimental values were measured in triplicate and expressed as mean ± standard deviations (SD). All data were analyzed by using SPSS software version 23. In order to determine the difference in the means, one-way analysis of variance (ANOVA) was carried out. The differences in the means were considered significant at 95% confidence limits ($p < 0.05$).

RESULT

Total Phenolic Contents in *Piper sarmentosum*

Figure 1 describes the total phenolic contents for the free, soluble and insoluble extracts of *P. sarmentosum* sample. Insoluble bound phenolic extract showed the highest TPC value (1.54 ± 0.04 mg GAE/g DW) followed by soluble phenolic extract (1.13 ± 0.10 mg GAE/g DW) and free extract (0.57 ± 0.06 mg GAE/g DW) ($p < 0.05$). TPC values of *P. sarmentosum* can be ranked as follows: bound insoluble > bound soluble > free. By using one-way analysis of variance (ANOVA) and Tukey's Test for post hoc test, it can be seen that there was a significant difference ($p < 0.05$) between free, soluble and insoluble extracts of *P. sarmentosum*.

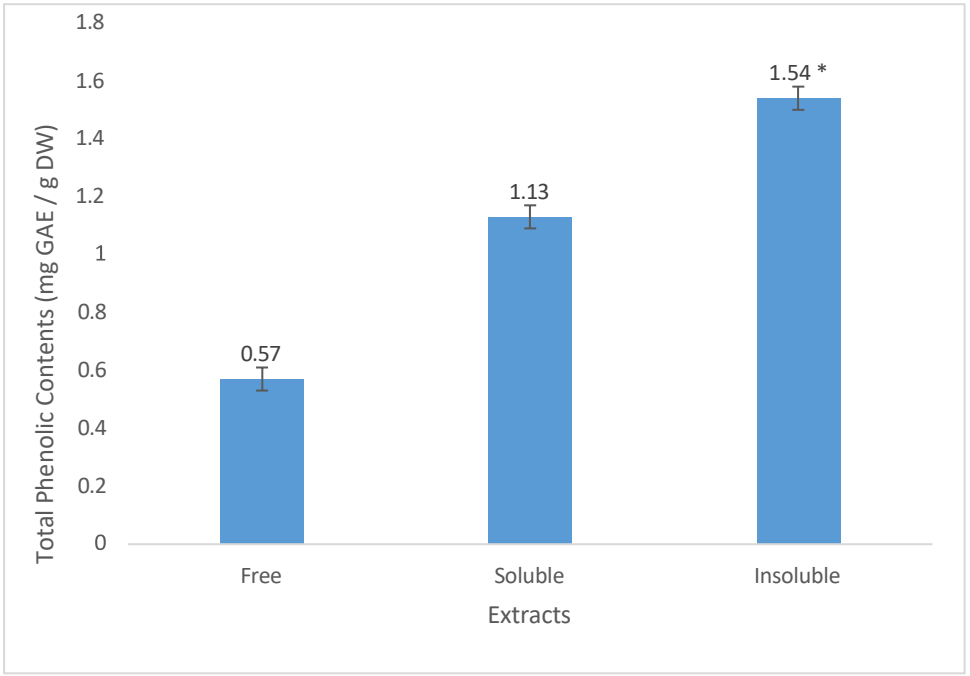

Figure 1: TPC of three different extracts of *P. sarmentosum*

Antioxidant Activity of *Piper sarmentosum*

Figure 2 shows the radical scavenging capacity of free, soluble and insoluble extracts of *P. sarmentosum*. At the concentration of 30 mg/ml, the soluble phenolic fraction has expressed the highest radical scavenging capacity (76.57± 4.12%) followed by insoluble (69.79± 2.33%) and free extracts (58.15± 4.44%). The antioxidant scavenging capacity can be ranked as soluble > insoluble > free.

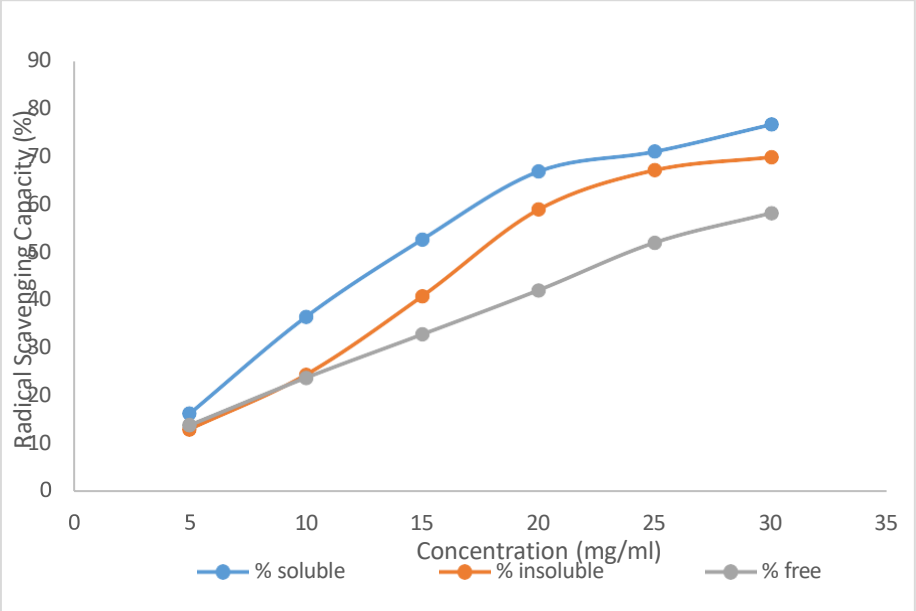

Figure 2: Percentage of radical scavenging capacity against concentration of extracts of *P. sarmentosum*

IC_{50} value (the concentration needed to scavenging 50% DPPH free radicals) was calculated from the concentration against scavenging activity graph. 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 extracts. The highest amount of IC_{50} in the free bound phenolic indicates weak reducing power which contain lower amount of radical scavenging capacity.

Table 1: IC_{50} value in *P. sarmentosum* extracts

Extracts	**IC_{50} (mg/ml)**
Free	24.05 ± 3.81 *
Soluble	16.17 ± 1.84 *
Insoluble	18.49 ± 1.92

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

It can be seen that there was a significant difference between each extracts of *Piper sarmentosum* and antioxidant inhibition (ANOVA analysis). It shows that different types of extracts contribute to the different radical scavenging capacity and also inhibition concentration. Post hoc comparisons using Tukey's test examined the significant difference between free, soluble and insoluble bound phenolic. From the comparison, the results showed that free and soluble bound phenolic were statistically significant difference ($p < 0.05$). Meanwhile, there no significant difference was observed between free and insoluble bound and also soluble and insoluble bound.

DISCUSSION

Total Phenolic Contents in *Piper sarmentosum*

There were three different extracts of *P. sarmentosum* that have been analyzed (free extracts, bound soluble extracts and bound insoluble). TPC values of *P. sarmentosum* can be ranked as follows: bound insoluble > bound soluble > free. This pattern is in line with the study conducted by Singh *et al*. (2013), that showed the TPC value of Moringa oleifera seed flour was higher in the bound extract as compared to the free extracts. In addition, study conducted by Ongphimai *et al*. (2013) showed that insoluble bound phenolic was higher compared to the free extracts in Thailand's fruit extracts. These results showed that most of the phenolic compounds are in bound form such as in esters form or glycosides form.

Antioxidant Activity of *Piper sarmentosum*

The *P. sarmentosum* extracts were able to reduce the stable DPPH to the yellow colored of diphenyl picryl hydrazine. Hence, the purple color of 2,2-diphenyl-1-picryl hydrazyl (DPPH) has been reduced to a, a-diphenyl-B-picrylhydrzaine (yellow colored). Scavenging activity increased with the increase in the concentration of the extracts (Singh et al; 2013). The antioxidant scavenging capacity can be ranked as soluble > insoluble > free. Although the TPC was higher in bound insoluble extract, the radical scavenging capacity was higher in bound soluble extracts. It may be due to the slow rate of reaction between DPPH and the phenol molecules of insoluble extracts compared to the soluble extracts. (Ongphimai et al; 2013). The use of acid hydrolysis treatment can decrease the content of the phenolic acids in the extract (Krygier *et al*., 1982) but the addition of a metal chelator like (EDTA) during the alkaline hydrolysis treatment can help to prevent the degradation of phenolic acids (Nardini *et al*., 2002). Other factors might influence the antioxidant activity of the plant extract such as temperature, the concentration of the antioxidant and the pH used during extraction process (Gazzani *et al*., 1998). However, this study was only focusing on the total phenolic compound (free, soluble and insoluble) that presence in the sample extract thus it is not known whether others factor might influence the antioxidant activity of the plant. These reports further affirmed that the total phenolic content does influence the antioxidative capability of the plant extract.

IC_{50} value (the concentration needed to scavenging 50% DPPH free radicals) was calculated from the concentration against scavenging activity graph. The IC_{50} is inversely proportional to radical scavenging capacity. It can be seen that there is a significant difference between each extracts (free and bound forms) of *Piper sarmentosum* and antioxidant inhibition (ANOVA analysis). It shows that different types of extracts (free and bound) contributed to the different radical scavenging capacity and also inhibition concentration.

CONCLUSION

In this study, different forms of phenolic have shown different amount of total phenolic contents and antioxidant activity. Free and bound extracts of *Piper sarmentosum* demonstrated different TPC values and antioxidant activities.

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