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ORIGINAL ARTICLE

Phenolic profiling of selected herbal plants: free, soluble bound and insoluble bound phenolics and their antioxidant activities

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Running short title: Phenolic profiling in herbal plants

ABSTRACT
This study was conducted to profile selected phenolic acids, total phenolic content and antioxidant activities of five herbal plants namely Citrus hystrix, Coriandrum sativum, Murraya koenigii, Polygonum minus and Ocimum basilicum in three different phenolic fractions (free, soluble bound and insoluble bound). The phenolic content was quantified using Folin-Ciocalteu method and phenolic acids were separated using C6-phenyl column High Performance Liquid chromatography. The antioxidant properties were determined using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) and ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) assays. The result showed that gallic, caffeic, ferulic, sinapinic and p-coumaric acids were detected in distinctive proportion. Bound forms of phenolic acids (soluble and insoluble bound) have high phenolic contents as compared to free phenolic. P. minus and O. basilicum demonstrated higher total phenolic content and showed promising antioxidant activities .The antioxidant properties can be correlated with the presence of phenolic acids in the extracts. To conclude, the herbal plants have the potential in reducing ability due to their strong electron-donating effects.
Keywords; *Free phenolic, bound phenolic, phenolic acids, herbal plants, antioxidant, free radical*

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INTRODUCTION

Non-communicable diseases (NCDs) including cardiovascular, diabetes mellitus, cancer and chronic respiratory diseases are accounted for 70% of global death as reported by the World Health Organization (WHO) in 2015. Other than physical inactivity and unhealthy diets, free radicals are one of the risk factors that initiate the progression of these deadly diseases. Free radicals are fundamental in cellular metabolism and defense system of the body. However, exposure to radiation, industrial solvent, air pollutant, cigarette smoke and heavy metal triggered excessive production of free radicals [1]. Overabundance of free radicals creates a harmful condition called oxidative stress that causes damage at the molecular level of the body. Nevertheless, the damaging effect of oxidative stress can be reversed by a mechanism involving antioxidants. Antioxidants are stable molecules act through its radical scavenging mechanism to inhibit or delay the oxidative chain reaction. Superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) are examples of enzymatic antioxidants presence in the body, whilst non-enzymatic antioxidants can be obtained through dietary supply [2].

Interestingly, the consumption of fruits, vegetables, cereal and grains could provide natural sources of antioxidants that exist in different forms including micronutrients and phytochemicals. Herbal plants are one of the antioxidant sources that have attained considerable attention in the primary health care. Of relevance, herbal plants are readily available, affordable and culturally acceptable which increased public acceptance and scientific evidences linking plants and their potential health benefits. More importantly, they contain many bioactive compounds especially polyphenols where its function in ameliorating

diseases and illness are well studied. Redox activities of phenolic compounds showed greater potential as free radical scavenger and hence promising biological properties including anti-microbial, anti-diabetes, anti-fungal and anti-cancer [3,4,5,6].

It is well known that phenolic compounds exist naturally in free and bound forms. Free form of phenolics can be found in the vacuole of plant cell, meanwhile majority of the polyphenols are in bound form and covalently bond to the cell wall structure including cellulose, hemicelluloses, pectin and structural proteins. Shahidi and Yeo (2016) reported that bound phenolics in fruits, vegetables and legumes are accounted for 20%-60% of total phenolics [7]. Free phenolics are extractable using organic or aqueous solvent. In contrast, bound phenolics need special acid, alkaline or enzyme treatments to liberate phenolic compounds from the plants [8].

Most studies of *in-vitro* antioxidant activities from herbal plants have only focused on quantification of total phenolic contents [9,10]. However, the literature of free and bound phenolics composition in plants is still limited. Free phenolic can be easily absorbed into the circulation, while bound phenolics need to be hydrolyzed by the action of intestinal enzymes or colonic microbiota. Thus, the extraction and quantification of bound phenolic could not be neglected as it may underestimate the total phenolic content and biological properties contribute by those phenolics in the food consumed. Thus, this research conducted to determine total phenolic content (TPC), profile selected phenolic acids and their potential in pharmacological properties as radical scavenger in five local plants consumed by Malaysian.

MATERIAL AND METHODS

Materials

Solvents (methanol, diethyl-ether and hexane) were analytical grade. DPPH (2, 2-Diphenyl-1-picrylhydrazyl) (SIGMA, Germany), ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) (Amresco, Ohio) and Folin-Ciocalteu reagent (MERCK, Germany) were used as standard.

Procedure

Samples preparation

Sweet basil (*Ocimum basilicum*), Curry (*Murraya koenigii*), Kaffir lime (*Citrus hystrix*), Coriander (*Coriandrum sativum*) and Kesum (*Polygonum minus*) leaves were purchased from local market in Kuantan, Pahang, Malaysia. The leaves were chopped into small pieces and freeze-dried for a week. The dried leaves were finely powdered and stored in -80 ºC freezer until further analysis.

Extraction of free phenolic fraction

The method was adapted from Dvorakova *et al*. (2008) with slight modifications [11]. A gram of powdered samples were weighted and added to 20ml of 80% methanol (80:20, v/v). The samples were further mixed by sonication for one hour at room temperature. This process was repeated twice under the same condition. The supernatant was filtered to get rid of all the impurities, while the residue was kept for insoluble-bound phenolic extraction. Methanol was removed from the filtered samples and 30 ml of diethyl-ether was used to further extract the samples. The bottom layer was set aside and kept for soluble bound phenolic extraction, while the supernatant was evaporated to dryness. The residue was dissolved in 10 ml of absolute methanol and labeled as free phenolic fraction.

Extraction of soluble bound fraction

The aqueous layer from the extraction with diethyl ether was treated with 20ml of 4M sodium hydroxide (NaOH) and stored at room temperature for 2 hours. The extract was acidified to pH 2 by using 6M hydrochloric acid (HCl). Extract was then partitioned with 30 ml diethyl ether and the organic phase was taken and evaporated to dryness. The residue was dissolved in 10 ml of absolute methanol and labeled as soluble bound fraction.

Extraction of insoluble bound fraction

The residue kept from the methanol extraction was further hydrolyzed using 10ml of 4M NaOH and stored at room temperature for an hour. The extract was acidified to pH 2 by using 6M hydrochloric acid (HCl) and centrifuged for 10 minutes at 4380 rpm. The supernatant was collected and further extract with 15 ml hexane. The hexane layer was removed using rotary evaporator and the sample was further extracted with 30 ml diethyl ether. The obtained supernatant was evaporated to dryness. The residue was dissolved in 10 ml of absolute methanol and labeled as insoluble bound fraction.

HPLC analysis for phenolic compounds

HPLC analysis was carried out using Perkin Elmer HPLC coupled with photo-diode array (PDA) detector. Phenolic compounds separation was performed on reversed phase Phenomenex C6-phenyl column (250 mm x 4.6 mm ID, 5 µm particles) in isocratic elution mode. Column temperature was maintained at 25^{o}C. Identification of phenolic compounds was based on retention times in comparison with standards and the quantification was carried out using the external standard method. Samples were filtered prior to

injection with injection volume 5 µl. HPLC analysis was done according to El-Achi *et al*. (2014) with slight modifications [12]. Mobile phase used consisted of methanol (solvent A) and 1% formic acid in water (solvent B) (80:20). The flow rates of gallic, caffeic and *p*-coumaric acids were kept at 0.8 ml/ min and monitored at 270 nm, whereas ferulic and sinapinic acids were monitored at 320 nm at 0.7 ml/ min. The concentration of phenolic compounds presented in the samples was expressed as µg/g of dry weight.

Total Phenolic Content Assay

Total phenolic content (TPC) assay was done using Folin-Ciocalteu method with slight modifications [9]. Samples were diluted with methanol in a range of 3 to 50 mg/ml. Samples were mixed with 10% Folin-Ciocalteu (FC) reagent and left to stand at room temperature for 5 minutes. Five percent of sodium carbonate (w/v) was added to the mixture and incubated for one hour at room temperature. The absorbance was read at 760 nm using microplate reader (Infinite M200 Pro). Gallic acid was used as a standard and the reading was expressed as gallic acid equivalents mg GAE /g of extract.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH assay was carried out as described by Azlim Almey *et al*. (2010) with slight modifications [9]. Extracts concentrations were in a range of 1.56 to 50 mg/ml. Gallic acid was used as a standard. DPPH reagent (160 µl) was added into 40 µl of samples and allowed to stand for 30 minutes. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Infinite M200 Pro). All tests and analyses were run in triplicates and averaged.

2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay

ABTS assay was conducted as described by Li *et al*. (2012) with slight modifications [13]. The samples concentration ranged from 1:1 to 1:32 dilution factor. Samples (50 µl) were mixed with ABTS solution (200 µl).The mixture was stand in a dark condition and the absorbance was measured at 734 nm after 30 minutes using spectrophotometer (Infinite M200 Pro). All tests and analyses were run in triplicates and averaged.

Statistical analysis

All analyses were run in triplicate and averaged with standard deviation. Statistical analysis was performed according to the SPSS version 20.0. Analyses of variance were performed using the one-way ANOVA procedure. A value of $p < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Phenolics profile by HPLC

The concentrations and types of phenolic acids detected in selected herbal plants are shown in Table 1 varied considerably. Analysis by HPLC revealed that *C. sativum* and *P. minus* were detected with all five phenolic acids in all fractions. On the other hand, gallic acid was not present in free and soluble bound fractions (*M. koenigii* and *C. hystrix*, respectively) and bound fractions (soluble and insoluble) of *O. basilicum*. *C. hystrix* contained caffeic acid as the major compound extracted in their free from, whilst bound fractions (soluble and insoluble) were highest in sinapinic acid ($p<0.05$). Caffeic acid was a predominant compound detected from all fractions of *C. sativum*, where insoluble bound fraction showed significantly higher concentration, followed by free and soluble bound fractions ($p<0.05$). This result was in agreement for the study done on the aqueous extract of *C. sativum* by De Almeida Melo *et al*. (2005), where the presence of caffeic acid was detected in all leaves fractions with the highest concentration in fraction I (4.34 µg/ml, ethyl ether: petroleum ether: methanol: water, 20:45:35:10 v/v) [14]. In addition, the seed of *C. sativum* has also been shown to contain caffeic acid with value of 0.19 ± 0.01 mg/g of seed extract [15].

It is clearly seen that free and soluble bound fractions of *M. koenigii* afforded the highest content of sinapinic acid in significant amount ($p<0.05$). In contrast, caffeic acid was the most prominent compound detected from insoluble bound fraction as compared to other phenolic acids from the same plant. Zhang *et al*. (2011) reported sinapinic acid amounted 0.79 ± 0.18 mg/g of dry weight was detected in the methanolic extract of *M. koenigii* leaves [16]. *P. minus* extract have demonstrated higher amount of *p*-coumaric acid in free phenolic fraction ($p<0.05$), whilst gallic acid was higher in bound fractions (soluble and insoluble bound) ($p<0.05$). Qader *et al*. (2012) found significant amounts of gallic acid and coumaric acid in *P. minus* extract (ethyl acetate: methanol, 1:1 v/v) [17]. Caffeic acid and its derivatives, chlorogenic acid, rosmarinic acid, lithospermic acid, chicoric acid and caftaric acid have a wide distribution in *O. basilicum* [18,19]. Upon the hydrolysis of the extract, the cleavage of ester linkage in caffeic acid derivatives could release high concentration of caffeic, ferulic and coumaric acid. This could be the possible explanation for the highest release of bound forms of caffeic acid in comparison with the free form. However, no significant differences were observed between soluble bound and insoluble bound fractions of *O. basilicum* ($p>0.05$).

Table 1 Phenolics composition of herbal plants in three phenolic fractions

Phenolic compounds	Phenolic fractions (µg/g of dry weight)		
C. hystrix	Free	Soluble bound	Insoluble bound
Gallic acid	5.95 ± 4.77[a]	nd	0.41 ± 0.57[a]
Caffeic acid	**87.34 ± 103.94[a]**	43.91 ± 1.78[a]	14.60 ± 0.31[a]
p-coumaric acid	62.07 ± 77.91[a]	65.53 ± 4.78[a]	12.05 ± 0.44[a]
Ferulic acid	1.53 ± 0.79[a]	57.83 ± 5.01[b]	12.72 ± 0.63[b]
Sinapinic acid	3.10 ± 1.60[a]	**117.69 ± 10.19[b]**	**25.89 ± 1.29[a]**
C. sativum	Free	Soluble bound	Insoluble bound
Gallic acid	0.13 ± 0.18[a]	3.43 ± 0.92[a]	3.18 ± 4.50[a]
Caffeic acid	**34.64 ± 9.39[a]**	**158.97 ± 3.26[a]**	**461.41 ± 94.66[b]**
p-coumaric acid	22.56 ± 7.04[a]	44.68 ± 6.41[a]	39.12 ± 3.92[a]
Ferulic acid	2.83 ± 0.98[a]	24.39 ± 2.20[ab]	46.06 ± 8.76[b]
Sinapinic acid	5.76 ± 1.99[a]	49.65 ± 4.48[ab]	93.73 ± 17.83[b]
M. koenigii	Free	Soluble bound	Insoluble bound
Gallic acid	Nd	27.84 ± 34.40[a]	6.15 ± 0.75[a]
Caffeic acid	72.97 ± 85.16[a]	48.70 ± 27.84[a]	**111.36 ± 2.73[a]**
p-coumaric acid	107.09 ± 15.07[a]	53.89 ± 16.19[ab]	48.78 ± 3.71[b]
Ferulic acid	133.24± 11.17[a]	41.98 ± 6.66[b]	52.36 ± 2.84[b]
Sinapinic acid	**271.17 ± 22.74[a]**	**85.43 ± 13.55[b]**	106.57 ± 5.79[b]
P. minus	Free	Soluble bound	Insoluble bound
Gallic acid	59.36 ± 19.84[a]	**391.83 ± 37.43[b]**	**131.36 ± 8.95[a]**
Caffeic acid	49.30 ± 1.57[a]	40.24 ± 12.98[a]	91.57 ± 3.39[b]
p-coumaric acid	**98.34 ± 12.48[a]**	52.44 ± 19.56[ab]	38.94 ± 1.70[b]
Ferulic acid	27.33 ± 4.85[a]	40.09 ± 0.97[a]	15.87 ± 10.88[a]
Sinapinic acid	16.09 ± 0.83[a]	81.60 ± 1.98[b]	32.30 ± 22.15[ab]
O. basilicum	Free	Soluble bound	Insoluble bound
Gallic acid	0.62± 0.88[a]	nd	nd
Caffeic acid	**18.99 ± 0.42[a]**	**442.60 ± 10.78[b]**	**342.21 ± 63.30[b]**
p-coumaric acid	15.84 ± 4.81[a]	72.92 ± 24.09[ab]	75.67 ± 1.85[b]
Ferulic acid	9.65 ± 2.13[a]	24.05 ± 0.52[a]	82.78 ± 22.56[b]
Sinapinic acid	12.44 ± 5.84[a]	47.45 ± 1.06[a]	168.47 ± 45.91[b]

Data are expressed as means ± standard deviation (n=2) determined by HPLC analysis. In each row, different superscript letter indicates significant different (p<0.05) in ANOVA test. nd Not detected

The release of free polyphenols was affected by the polar nature of solvent, whereas bound forms of polyphenols were liberated upon acid and alkaline hydrolysis [11]. In this study, the mixture of methanol and water was used to extract phenolic acids. Methanol was used to extract low molecular weight of phenolic compounds, while aqueous acetone was used in the extraction of high molecular weight compounds [20]. Interestingly, more than 50% of phenolic acids in the samples were found in bound forms (soluble and insoluble) (*C. hystrix*; *C. sativum*; *P. minus*; *O. basilicum*, 68.7%; 93.3%; 78.5%; 95.6%, respectively). Previous studies have reported higher amount of bound phenolic in several samples of barley, malt, araticum fruits, apple and beer [11,21,22,23]. These results may be explained by the fact of further hydrolysis using acid and alkaline to release the bound forms. Phenolic compounds easily release into the organic solvent in acidic environment as it helped to neutralize the compounds and break the glycosidic bonds. Meanwhile, alkaline treatment aids in the breaking of ester and ether bonds attaching phenolic acids to the cell wall [8,24].

Total phenolic content (TPC)

The quantification of the total phenolic content of plants samples were expressed as mg GAE/g (Figure 1). The TPC of all extracts ranged from 0.12 to 2.47 mg GAE/g. *P. minus* has demonstrated higher phenolic content in free phenolic and soluble bound fractions with concentration of 2.47 ± 0.57 mg GAE/g and 1.75 ± 0.57 mg GAE/g, respectively. Meanwhile, *O. basilicum* has predominant amount of phenolic extracted from insoluble bound fraction (1.99 ± 0.41 mg GAE/g, p<0.05). The results clearly showed that *C. hystrix* possessed the lowest amount of phenolic content in each fraction. To sum up, the *O. basilicum*, *C. sativum*

and *C. hystrix* were comprised of 70.9%, 82.7% and 64.3% phenolic released from bound fractions (soluble bound and insoluble bound), respectively.

The total phenolic of *P. minus* reported was in a good agreement with previous study done by Maizura *et al*., (2011) which demonstrated highest TPC in *P. minus* (165.34 mg GAE/100 g extract), followed by ginger (101.56 mg GAE/ 100 g extract) and turmeric (67.89 mg GAE/ 100 g extract) [10]. Azlim Almey *et al*. (2010) found the highest amount of phenolic content in *P. minus* as compared to *M. koenigii*, *C. hystrix* and *P. odorus* methanolic and ethanolic extracts [9]. Some assays might over quantify the phenolic content due to the presence of carbohydrates, ascorbic acid and amino acid that could affect the reduction of Folin-Ciocalteu reagent. Thus, antioxidant assays such as DPPH and ABTS were selected as a second response variable due to its related property to the total phenolic content.

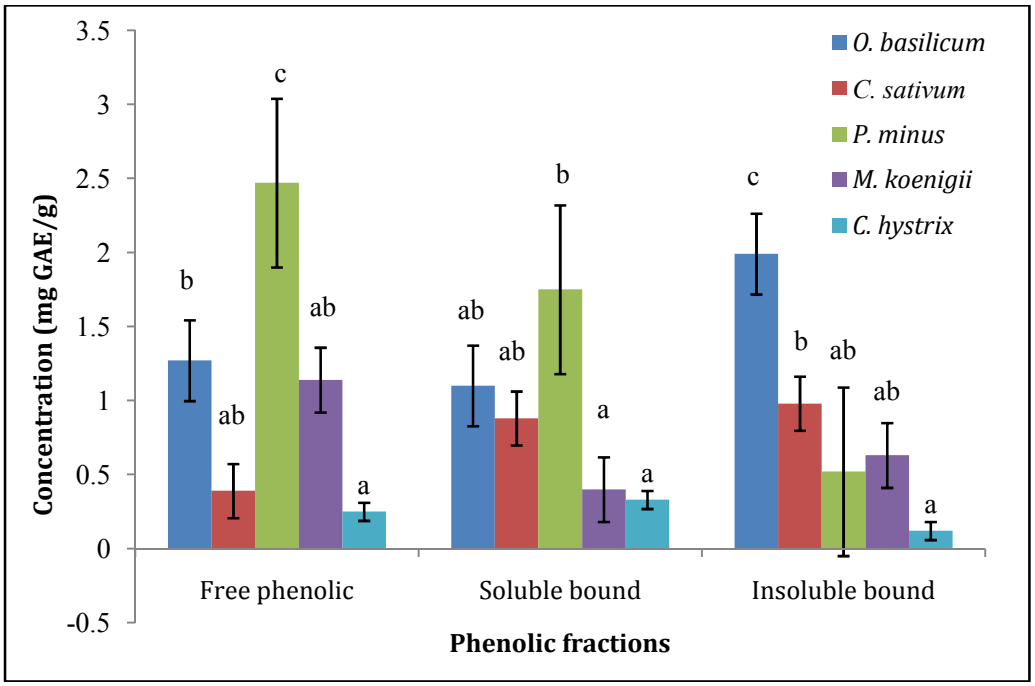

Figure 1 Total phenolic content of free, soluble bound and insoluble bound fractions in herbal plants at 6.25 mg/ml. Bars with different letters in each fractions are significant different (p<0.05) in ANOVA test.

Antioxidant properties

The antioxidant activities of samples were evaluated in, all five extracts from three different fractions and have shown significant different in terms of radical scavenging capacity ranged from 3.66% to 91.34% (p<0.05) (Table 2). In the case of DPPH assay, free phenolic extract of *P. minus* demonstrated the highest radical scavenging activities and significantly different in comparison to *C. sativum* and *C. hystrix* (p<0.05). In fact, scavenging activity of *P. minus* extract was more than 20-fold higher than that of *C. hystrix*. Likewise, soluble bound fraction also showed the same trend, where *P. minus* had significantly higher percentage of radical scavenging activities when compared to *C. hystrix* (p<0.05). On the other hand, for insoluble bound fraction, radical scavenging activity was significantly the highest in *O. basilicum* when compared to other plant extracts (p<0.05). In addition, *C. hystrix* had the lowest radical scavenging activity when compared to *O. basilicum*, *C. sativum*, *P. minus* and *M. koenigii* (p<0.05).

In the ABTS assay, antioxidant activities of all extracts were varied from 5.41% to 94.9%. A similar trend was observed in both assays (DPPH and ABTS). Free phenolic fraction of *P. minus* showed an effective antioxidant activity, significantly higher as compared to *C. sativum* and *C. hystrix* (p<0.05). Similarly, the most promising radical scavenger was demonstrated by *P. minus* extract when compared to *M. koenigii* and *C. hystrix* for the soluble bound fraction (p<0.05). For insoluble bound fraction, the radical scavenging activity of *O. basilicum* was found higher than the other plant extracts (p<0.05). *C. hystrix* had the lowest radical scavenging activity in comparison to the *O. basilicum*, *C. sativum* and *P. minus* (p<0.05). These findings did support the TPC assay's result, where *P. minus* and *O. basilicum* exhibited high TPC compared to others.. There was a positive significant correlation between total phenolic content and antioxidant activities (DPPH, r = 0.865; ABTS, r = 0.857) according to the Pearson correlation test.

Table 2 Antioxidant properties of free, soluble bound and insoluble bound fractions in plants at 6.25 mg/ml.

Assay	Leaves extracts **Free phenolic fraction**				
	C. hystrix	*C. sativum*	*M. koenigii*	*P. minus*	*O. basilicum*
DPPH radical inhibition (%)	4.37 ± 0.20^{cb}	7.00 ± 5.06^{b}	70.57 ± 21.93^{a}	**91.34 ± 0.94^{ad}**	68.10 ± 16.15^{a}
ABTS radical inhibition (%)	8.62 ± 2.12^{b}	17.64 ± 5.06^{b}	68.41 ± 15.12^{a}	**85.87 ± 3.61^{a}**	60.43 ± 19.72^{a}
	Soluble bound fraction				
	C. hystrix	*C. sativum*	*M. koenigii*	*P. minus*	*O. basilicum*
DPPH radical inhibition (%)	4.69 ± 2.07^{bd}	40.23 ± 39.36^{ab}	19.66 ± 6.82^{ad}	**90.31 ± 2.41^{ac}**	68.44 ± 15.04^{a}
ABTS radical inhibition (%)	12.66 ± 3.89^{c}	41.70 ± 11.12^{ac}	27.83 ± 10.29^{c}	**94.85 ± 4.13^{ab}**	66.37 ± 24.93^{ab}
	Insoluble bound fraction				
	C. hystrix	*C. sativum*	*M. koenigii*	*P. minus*	*O. basilicum*
DPPH radical inhibition (%)	3.66 ± 3.13^{c}	53.93 ± 19.71^{b}	32.65 ± 7.21^{b}	56.91 ± 6.28^{b}	**86.42 ± 2.90^{a}**
ABTS radical inhibition (%)	5.41 ± 2.40^{b}	45.70 ± 26.10^{a}	28.63 ± 8.93^{ab}	40.98 ± 7.60^{a}	**77.36 ± 4.38^{c}**

Values are expressed as mean (n=3) ± standard deviation. Values with different superscript letter within each rows are significant different at the level $p<0.05$ in ANOVA test.

The presence of multiple hydroxyl groups in gallic and caffeic acids structures detected mostly from *P. minus* and *O. basilicum*, respectively could be a key determinant for the highest antioxidant activities shown by both samples. The redox properties of phenolics in combating free radicals depend on the number and position of hydroxyl group attach at benzene ring structure, where hydroxyl group could donate hydrogen atoms or electron to scavenge free radicals. Moreover, the *ortho* position of hydroxyl groups in both gallic acid and caffeic acid structures aid in the resonance stabilization, thus lowering bond dissociation enthalpy and contribute to the high antioxidant capacity [25]. Apart from the structural component of antioxidant agent, the degree of its activity can also be significantly affected by solvent polarity, temperature, pH, type and concentration of reactive species [26,27]. Arruda *et al*. (2018) also points out that synergistic or antagonistic interaction between distinctive compounds in the samples could give significant effect to the antioxidant activity [21].

CONCLUSION

The results of this study indicate that the phenolic profile of free, soluble bound and insoluble bound fractions and their antioxidant activities of all samples are varies. Majority of the phenolic compounds from *O. basilicum*, *C. sativum*, *P. minus* and *C. hystrix* were in bound form. All plants, especially *O. basilicum* and *P. minus* show the promising antioxidant effect. Considering this information, these plants could be potentially exploited and commercialized in the future alternatives medicines in combating free-radical related diseases.

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