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Antioxidants Content and Activity of Polyphenol-Rich Mixtures

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Abstract: This study aimed to determine antioxidants content and antioxidant activity of Polyphenol-Rich Mixture (PRM) samples containing garlic, ginger, lemon, apple cider vinegar and honey. Three PRM samples (raw, cooked and commercial) were subjected to Folin-Ciocalteu reagent and aluminium chloride colorimetric assays for determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) respectively. Antioxidant activity was determined based on DPPH free radical scavenging and Ferric Reducing Antioxidant Power (FRAP) assays. TPC and TFC of the three samples were significantly different ($p<0.05$) where cooked and commercial PRM had the highest antioxidants content. Similarly, cooked and commercial PRM showed the lower EC_{50} values indicating both samples possess higher antioxidant activity compared to raw PRM. The cooked and commercial PRM also had higher FRAP values which showing a significant difference between the PRM samples ($p<0.05$). Pearson correlation analysis demonstrated high negative correlations between DPPH scavenging activity and total phenolics (TPC and TFC) with $r = -0.855$, $r = -0.829$, respectively. FRAP values of the PRM samples were also positive and highly correlated with TPC and TFC ($r = 0.995$, $r = 0.988$, respectively). This finding clearly indicated that cooked and commercial PRM possess high antioxidants content and antioxidant activity and can be considered as potential natural antioxidant beverages for prevention of chronic diseases.

Key words: Antioxidant assay, polyphenol-rich mixture, *Allium sativum*, *Zingiber officinale*, citrus×limon, apple cider vinegar, honey

INTRODUCTION

Reactive oxygen species and other free radicals are produced in our body as a result of metabolism and environmental factors (Pham-Huy *et al*., 2008). Overload of free radicals will cause oxidative stress which indicates redox imbalance between oxidant and antioxidant (Valko *et al*., 2016). Subsequently, this lead to many pathological conditions such as cardiovascular diseases, cancer, hypertension is chemia, diabetes, neurodegenerative diseases (Alzheimer's and Parkinson's diseases) rheumatoid arthritis, asthma and aging (Valko *et al*., 2016; Giacco and Brownlee, 2010; Reuter *et al*., 2010; Sahiner *et al*., 2011). According to World Health Organization in the year 2015, Non-Communicable Diseases (NCDs) killed 36 millions people each year. The four main NCDs that cause premature death are cardiovascular diseases (17.5 million people annually) cancers (8.2 million) respiratory diseases (4 million) and diabetes (1.5 million) (WHO., 2015).

Antioxidant is any substance that delays, prevents or reduces oxidative damage to a target molecule with its principle of inhibiting initiation and propagation of oxidizing chain-reaction (Gutteridge and Halliwell, 2010; Ismail *et al*., 2004) Phenolic compounds are natural antioxidants found in spices, fruits and vegetables (Khatun *et al*., 2006). Antioxidants exist in both free and bound forms in plant cells. The phenolic contents and antioxidant activities of fruits and vegetables could be underestimated if the bound phenolic compounds are not considered (Su *et al*., 2014). Therefore, several types of treatment such as acid, alkaline and enzymatic hydrolysis are performed to release the bound phenolic compound (Acosta *et al*., 2014). Moreover, spices mixture can either be consumed as raw or cooked food. Generally, process foods have been considered having a low nutrition value due to the loss of some nutritional components. However, there were studies showed that antioxidants from cooked vegetables are either remained unchanged or increased after cooking (Chang *et al*., 2006; Sultana *et al*., 2008).

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Spices such as garlic contain bioactive compounds (allicin, alliinase and alliin) where these bioactives have been reported to reduce ischemia and decrease lipid peroxidation (Gorinstein *et al*., 2006; Pedraza *et al*., 2007). Ginger is shown to have anti-inflammatory, anti-platelet and hypolipidemic effects due to its antioxidants (gingerol related compound and diarylheptanoids) (Nicoll and Henein 2009; Masuda *et al*., 2004). Lemon also contains numerous bioactive compounds (flavonoids such as hesperidin, flavonones and flavones) which have been beneficial for health (Gonzalez *et al*., 2010). Concurrent to this, apple cider vinegar contains anthocyanins, flavonols and flavanols which also shown to have multiple therapeutic effects such as prevention of hypertension and cardiovascular diseases (Setorki *et al*., 2010; Budak *et al*., 2014). Besides that, honey has also gained attention due to its therapeutic value contributed by phenolic acids and flavonoids (Farooqui and Farooqui, 2011). In recent year, there has been increasing trend of using indigenous plants as home remedies for various diseases due to synthetic drugs have been reported to have serious side effect (Javed *et al*., 2009). Despite from the therapeutic effect of individual ingredients, spices mixture also reported reducing the incidence of coronary diseases which might be due to its antioxidants properties (Javed *et al*., 2014).

Due to the increasing prevalence of NCDs and potential health benefits of local spices, three different formulations of a Polyphenol-Rich Mixture (PRM) were proposed for reducing oxidative stress and maintain good health of Malaysians. PRM is the spices mixture consists of garlic (*Allium sativum*) ginger (*Zingiber officinale*) lemon (Citrus×limon), apple cider vinegar and honey. All these ingredients are commonly available in local market and widely used by Southeast Asian community for cooking and for their medicinal purposes (Otunola and Afolayan, 2013). Hence, it is important to identify potential antioxidants content and antioxidant activity of the formulated PRM which is in raw and cooked forms after acid hydrolysis by organic acids from lemon and apple cider vinegar as well as heating using a high temperature for the cooked PRM. The present study focused on determination of total phenolics and antioxidant activity of raw, cooked and commercial PRM, as well as their correlations were determined.

MATERIALS AND METHODS

Samples: Samples (5 kg each) such as ginger, garlic and lemon were purchased from the local market at Serdang, Selangor. A bottle each of apple cider vinegar, honey and commercial PRM were purchased from an organic shop in Cheras, Malaysia.

Reagents and chemicals: All reagents and chemicals used were of analytical grade. Ethanol was purchased from Fisher Scientific (Leicestershire, UK) distilled water was prepared using a Favorit W4L water distillation system from Generico (Nottingham, UK). 2, 2-diphenyl-1-pircylhydrazyl (DPPH), sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) glacial acetic acid, 2, 4, 6-tri-(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCl) ferric chloride hexahydrate ($FeCl_3.6H_2O$) ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) aluminium chloride ($AlCl_3$) sodium nitrate ($NaNO_2$) and Tris-HCl (pH 7.0) were purchased from Sigma Chemical Co. (St. Loius, MO, USA). Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3) sodium hydroxide (NaOH) gallic acid (>95% purity) and quercetin (>95% purity) were purchased from Sigma-Aldrich (Steinheim, Germany).

Sample preparation: Ginger and garlic were sorted to remove dirt, peel and washed with tap water. The ginger and garlic were separately sliced into small pieces, made up to a half cup (125 mL) and then blended using a household blender with 62.5 mL to obtain its juice. Lemon was washed and squeezed to get about 125 mL of juice. Ginger and garlic juices were mixed with lemon juice and apple cider at a ratio of 1:1:1:1 and then homogenized to obtain a crude mixture. The mixture was filtered through a Whatman No. 1 filter paper. Lastly, 125 mL of honey was added to the filtered mixture. The final mixture was transferred into clean, dry and airtight glass bottle for further extraction and analysis. For cooked sample, a mixture of 125 mL of each juice of garlic, ginger, lemon and apple cider vinegar was boiled until half of its original portion for 60 min above a gas stove and left to cool to room temperature before addition of honey. Cooking is to hydrolyze bound phenolic compounds in the mixture to obtain full free phenolic compounds. The final mixture was packed into clean, dry and airtight glass bottle and stored in a refrigerator at -80°C. Both mixtures were named raw and cooked PRM. The commercial PRM was filtered using a Whatman filter study before freeze dried. All PRM samples were freeze dried using a Virtis benchtop freeze dryer before further extraction and analysis.

Sample extraction: The lyophilized PRM (5 g) was extracted using 100 mL of 80% aqueous ethanol in conical flasks sealed with foil and allowed to shake using an orbital shaker for 2 h at room temperature. The PRM

extract was subsequently filtered through a Whatman No. 1 filter paper to obtain a clear solution. The filtrate was transferred into a round bottom flask and evaporated at 40°C to remove ethanol using rotary evaporator. The aqueous extracts of PRM were determined for total phenolic content, total flavonoid content, DPPH radical scavenging assay and Ferric Reducing Antioxidant Power (FRAP) assay. The extracts were stored at -20°C. Triplicate extraction was performed for each PRM sample.

Determination of total phenolic content: Total phenolic content was determined by Folin-Ciocalteu reagent assay according to the method described previously (Su *et al*., 2014). About 0.5 mL of PRM extract (50 mg/mL) was added with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent into a test tube. The mixture was allowed to stand at room temperature for 5 min and then 2.0 mL (75 g/L) of sodium carbonate was added into the mixture. The tube was vortexed for 15 sec and allowed to stand in darkness for 30 min at 40°C. The absorbance was read at 765 nm using a spectrophotometer against a blank containing distilled water. All the tests were performed in triplicate. Total phenolic content of each sample was determined based on the equation obtained from the calibration curve plotted with different concentrations of gallic acid standard in 80% aqueous ethanol (10-100 μg/mL). The result was expressed as milligram of gallic acid equivalent per gram Fresh Weight (FW) of sample (mg GAE/g FW).

Determination of total flavonoid content: Aluminium chloride colorimetric assay was used to determine total flavonoid content of the extracts with a slight modification to the method described by Ghasemzadeh *et al*. (2010). About 2.0 mL of the diluted extract was mixed with 0.2 mL of 5% sodium nitrate in a volumetric flask and incubate at room temperature for 5 min. After 5 min, 0.2 mL of 10% aluminium chloride was added and mixed well. After 6 min, 2.0 mL of 1.0 M sodium hydroxide was added. Lastly, the volume was made up to 5.0 mL by addition of 80% ethanol and mixed well. After standing for 10 min at room temperature, absorbance of the reaction mixture was read at 430 nm using spectrophotometer against a blank containing 80% aqueous ethanol. All the tests were performed in triplicate analyzes. Total flavonoid content values were determined based on quercetin calibrate curve prepare from different concentrations of quercetin in 80% aqueous ethanol (10-100 μg/mL). Results were expressed as milligram of quercetin equivalent per gram fresh weight of samples (mg QE/g FW).

Determination of scavenging activity: DPPH radical scavenging method was performed according to Yan and Asmah with some modification (Yan and Rahmat, 2010). Sample extract (0.2 mL) of various concentrations (0.0976-50.0 mg/mL) prepared based on series dilution from stock solution was added to 0.8 mL of 100 mM Tris-HCl buffer (pH 7.0). Then, 1.0 mL of 0.5 mM DPPH which previously prepared in 95% ethanol was added to the mixture. The mixture was shaken vigorously and left to stand for 20 min at room temperature in a dark room. Absorbance was recorded using a spectrophotometer against a blank containing 95% ethanol. Scavenging effect on DPPH radical scavenging was calculated using the formula as follows:

$$\text{Scavenging activity}(\%)=\frac{\text{Absorbance (control)}-\text{Absorbance (sample)}}{\text{Absorbance (control)}}\times 100$$

For control, the reacting mixture contained 1.0 mL of 0.5 mM DPPH, 0.2 mL of 80% ethanol and Tris-HCl (pH 7.0). Gallic acid with various concentrations (0.0976-50.0 mg/mL) were used as standard calibration. All the tests were performed in triplicate. Sample concentration needed to reduce 50% of DPPH radical or EC_{50} value was calculated from the plotted graph of the sample.

Determination of antioxidant activity: Determination of antioxidant activity by FRAP assay was performed according to the reported method with slight modification for measuring the ferric reducing ability of sample extract (Ghasemzadeh *et al*., 2010). The FRAP reagent was prepared freshly by mixing 2.5 mL of TPTZ (10 mM in 40 mM hydrochloric acid), 2.5 mL of ferric chloride (20 mM in distilled water) and 25 mL of sodium acetate buffer (300 mM, pH 3.6 by adding 16.0 mL acetic acid and 3.1 g sodium acetate). About 50 μL of properly diluted sample extract was mixed with 3.0 mL of FRAP reagent. The reaction mixture was incubated at water bath at 37°C for 30 min in dark. The increase in absorbance was measured at 593 nm using spectrophotometer against a blank (distilled water). The change in absorbance at 0 min and 30 min was calculated. Ferrous sulphate solution with various concentration (1-1000 μM) was used to plot a calibration curve for quantification purpose. The antioxidant activity was expressed as milimolar of ferrous ion per gram fresh weight (mM Fe^{2+}/g FW). FRAP values of the samples were calculated according to formula as follows:

$$\text{FRAP value}=\frac{R\times DF\times V}{DW}\times 100\%$$

Where:

R = The x value obtained from the standard curve
DF = The Dilution Factor
V = The total volume of sample used
DW = Dry Weight of sample used for extraction

Statistical analysis: All data were determined in triplicate analyzes and analyzed using IBM SPSS Statistic Version 22. All results were expressed as mean± Standard Deviation (SD). One-way ANOVA and post-hoc test were performed to determine the differences in antioxidants content and antioxidant activity of raw, cooked and commercial PRM. Pearson's correlation test was conducted to determine correlation between antioxidants content and antioxidant activity of raw, cooked and commercial PRM. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Total phenolic content: Total Phenolic Content (TPC) of the sample extracts was quantified based on the linear equation obtained from the gallic acid standard calibration curve and expressed as gallic acid equivalent per gram fresh weight of samples (mg GAE/g FW). Comparison of TPC between raw, cooked and commercial PRM was presented in Table 1. Based on the results, TPC (mg GAE/g FW) was found to be in descending order according to different types of PRM: commercial PRM (12.94)>cooked PRM (7.52)>raw PRM (4.21). One-way ANOVA test revealed a significant difference between the PRM samples ($p<0.05$).

Previous study reported that TPC value of plant mixture in 75% aqueous ethanol extract in combination of garlic, peppers, lemon grass, kaffir lime fruit peels, nutmeg, shallot, cinnamon, camphor seed, cumin, turmeric, galangal and coriander seed are 173.87 mg GAE/100 g DW (Settharaksa *et al*., 2012) which is slightly lower than the cooked and commercial PRM samples (270.76 and 509.90 mg GAE/100 g DW, respectively). The difference in TPC between these two samples could be due to the different types of plant source used and also different extraction method applied. A previous study showed that yield of phenolics could be influenced by the type of extraction solvent, extraction time and extraction temperature used as well as solvent-to-sample ratio (Khoddami *et al*., 2013).

Applying 80% aqueous ethanol for extraction, the cooked and commercial PRM showed higher TPC values compared to raw PRM. This finding is in agreement with a previous study that there was a significant increase in TPC in the selected cooked vegetables compared to its raw form which is probably due to the cooking process softens and disrupts plant cell wall, as well as the destruction of complex phenolic (Otunola and Afolayan, 2013). The fact is further supported by another study which showed that hydrothermal treatment from 15-30 min was able to disrupt the plant cell membranes and cell walls (Dewanto *et al*., 2002). Hence, hydrolysis of complex phenolic compound into a simple form increases level of free phenolic compounds thereby lead to an increase in TPC.

Table 1: Antioxidants content of raw, cooked, and commercial PRM

Samples	TPC (mg GAE/g FW)	TFC (mg QE/g FW)
Raw PRM	4.21±0.16^{a}	2.18±0.01^{a}
Cooked PRM	7.52±0.50^{b}	4.19±0.0^{b}
Commercial PRM	12.94±1.67^{c}	8.56±0.07^{c}

Values are expressed as mean±standard deviation of three replicates. Mean values with different superscript lowercase letters ($^{a-c}$) indicate a significant difference at the level of $p<0.05$

Total flavonoid content: Total Flavonoid Content (TFC) of the sample extracts was quantified based on the linear equation obtained from quercetin standard calibration curve and expressed as quercetin equivalent per gram fresh weight of samples (mg QE/g FW). Comparison of TFC between raw, cooked and commercial PRM was presented in Table 1. Based on the results obtained, TFC of the PRM samples (mg QE/g FW) was found to be in descending order: commercial (8.56)>cooked PRM (4.19)>raw PRM (2.18). Analysis of one-way ANOVA found a significant difference between the PRM samples ($p<0.05$).

Similar to what has been reported for TPC, the literature shows a lower TFC (6.55 mg QE/g FW) in a plant mixture (ginger, garlic and chili pepper) than the commercial PRM sample determined in this study (Su *et al*., 2014). The variation in TFC could be due to the used of different in raw ingredient. Besides that, the high or low TFC could be influenced by the varieties and different parts of plant used (Ghasemzadeh *et al*., 2010). Absence or presence of some flavonoid compounds in different varieties and parts of the plants used could also be related to humidity, harvesting period and light intensity which caused different photosynthesis rates, hence, influence the flavonoids content (Shukri *et al*., 2011).

Moreover, use of 80% aqueous ethanol extraction gave a higher TFC in cooked and commercial compared to raw PRM. This finding is in agreement with a previous study that there was significant increased in TFC after heating the peel of kaffir lime at a temperature of 121°C (Settharaksa *et al*., 2012). Heat treatment increased releasing of free flavonoids in peel sample which could be due to the acid hydrolysis. Therefore, in this study, free flavonoids being released could also be resulted from acid hydrolysis using apple cider vinegar and lemon. Extraction of flavonoid compounds through acid hydrolysis at high temperature (80-100°C) is able to hydrolyze flavonoid glycosides and hence release free flavonoids (Dai and Mumper, 2010; Haghi and Hatami, 2010).

Table 2: Antioxidant activity of raw, cooked, and commercial PRM

Samples	DPPH EC_{50} (mg/mL)	FRAP (mM Fe^{2+}/g FW)
Raw PRM	49.30±3.9^{a}	39.48±0.10^{a}
Cooked PRM	26.25±3.7^{b}	66.36±0.20^{b}
Commercial PRM	19.12±4.1^{b}	103.50±0.20^{c}

Values are expressed as mean ± standard deviation of three replicates. Mean values with different superscript lowercase letters ($^{a-c}$) indicate a significant difference at level of $p<0.05$

Antioxidant activity: In this study, scavenging effect of PRM samples was evaluated using DPPH assay. The gallic acid standard curve plotted based on concentrations of 6.25-200 mg/mL was used for quantification of EC_{50} values of PRM samples. DPPH EC_{50} values were calculated as the amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50%. Among the three types of PRM sample, commercial PRM had the lowest EC_{50} (19.12 mg/mL), followed by cooked and raw PRM (26.25 and 49.30 mg/mL, respectively) as summarized in Table 2. A lower EC_{50} value indicates a higher antioxidant effect of PRM sample. Besides that, statistical analysis using one-way ANOVA showed a significant difference between the PRM samples ($p<0.05$). Based on the post-hoc result there was no significant difference in EC_{50} values between cooked and commercial PRM. As a result, cooked and commercial PRM were more efficient in scavenging DPPH free radical as compared to raw PRM. This efficiency could be due to thermal treatment to PRM sample increased the levels of free phenolics and flavonoids which in turn elevated the activity of DPPH radical scavenging. The DPPH radical scavenging effect might also be affected by TPC of PRM sample.

The result obtained from this study is similar to the finding reported by Shobana and Naidu where the extract of a spice mixture of garlic, ginger, cloves, cinnamon and pepper after boiling at the temperature of 100°C for 30 min showed a higher antioxidant activity than fresh sample mixture (Shobana and Naidu, 2000). Another study also reported that curry paste extract of a spice mixture of garlic, ginger, cloves, cinnamon and pepper showed a stronger scavenging effect (1.77 mg GAE/100 g) compared with the individual raw ingredients (0.28-1.01 mg GAE/100 g) (Seah *et al*., 2010). Moreover, boiling enables release of some bound phenolics and alters insoluble phenolic compounds to soluble phenolics which in turn increase the DPPH radical scavenging activity (Kim *et al*., 2006).

In this study, a standard calibration curve of ferrous sulphate in 80% aqueous ethanol extract at concentrations of 0.01-1.0 mM was used. The results were expressed as millimoles ferrous ions per gram fresh weight (mM Fe^{2+}/g FW). FRAP values of three types of PRM (raw, cooked and commercial) are shown in Table 1. From the results obtained, FRAP values of the three types of PRM ranged from 39.48-103.50 mM Fe^{2+}/g FW. The FRAP values are shown as follows: commercial>cooked>raw. On the other hand, one-way ANOVA showed that there were significant differences between the three types of PRM ($p<0.05$).

This study demonstrated that commercial PRM had the greatest antioxidant activity compared with cooked and raw PRM. Another study showed that dry spices mixture namely turmeric, pepper, cloves, ginger, basil, yellow justicia and sweet orange peel significantly inhibited lipid peroxidation and exhibited synergistic antioxidant activity (Odukoya *et al*., 2005). Seah *et al*. (2010) also, reported that a spice mixture of turmeric, garlic and chili had a lower FRAP value than turmeric and chili. It could be due to the reason that mixing of different spices may cause some chemical reactions which give different FRAP values. Therefore, the antioxidant activity of spices mixture depends on specific types of ingredient used. In this study, commercial PRM possessed a higher antioxidant activity than the other two PRM samples which might be due the commercial PRM contained added food preservative that increases shelf life and antioxidant activity of the product.

Correlation between total phenolics and antioxidant activity: In this study, Pearson's correlation coefficient test showed negatively high correlations between total phenolics (both TPC and TFC) and EC_{50} values from DPPH assay of PRM samples ($r = -0.855^{**}$ and $r = -0.829^{**}$, respectively) (Table 3). This finding agrees well with a previous study that showed a high correlation between total phenolics and free radical-scavenging power (Ghasemzadeh *et al*., 2010). On the other hand, a previous literature showed no correlation between total phenolics and antioxidant activity of spices mixture of ginger, garlic and pepper (Su *et al*., 2014). The no correlation might be due to the synergistic activity of antioxidant power of the spices mixture (Su *et al*., 2014; Seah *et al*., 2010).

The result also, showed positive correlations between total phenolics (both TPC and TFC) and FRAP values of PRM samples with $r = 0.995^{**}$ and $r = 0.988^{**}$, respectively (Table 3). On the contrary, a previous study showed no correlation between total phenolics and FRAP values of spice mixture of garlic, turmeric and chili (Seah *et al*., 2010). It could be due to the complicated chemical reaction of spices mixture which possessed different degrees of hydroxylation and conjugation of phenolic compounds and eventually leads to a variation of FRAP value (Pulido *et al*., 2000). Also, the higher antioxidant activity of phenolic compound generally involves more conjugated and numerous hydroxyl group.

Table 3: Correlation between antioxidants content and antioxidant activity of PRM samples

Antioxidant Contents /Antioxidant activity	TPC	TFC
DPPH radical scavenging activity (EC_{50})	r = -0.855**	r = -0.829**
Ferric reducing antioxidant power (FRAP value)	r = 0.995**	r = 0.988**

r refers to the correlation coefficient of Pearson test; **Significant difference at $p<0.01$

CONCLUSION

The result of this study indicated that there were significantly higher TPC, TFC, EC_{50} values of DPPH assay and FRAP values in cooked and commercial PRM compared to raw PRM. Acid hydrolysis in cooked and commercial PRM is able to release a more free phenolic and flavonoid compounds, thus, increased antioxidant activities in the PRM. High correlations were also determined between total phenolics and antioxidant activities of PRM samples. Although, this study found a high antioxidants content and strong antioxidant activities for all the PRM samples, there is a need to investigate the effect of the cooking processes such as cooking temperature, pH values and source of raw ingredients towards antioxidant properties of PRM. The mechanism of these antioxidants in the prevention of diseases is also unknown.

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