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Antioxidant Levels and Activities of Selected Seeds of Malaysian Tropical Fruits

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ABSTRACT

The aims of this study are to determine and compare the antioxidant levels and activities (i.e. primary and secondary) between selected seeds of Malaysian tropical fruits - guava (*Psidium guajava*), mango (*Mangifera indica* L.) and papaya (*Carica papaya* L.). Seeds are among byproducts from the processing of fruits-based products. Instead of discarding seeds as waste, seeds with high potential as antioxidants could be utilised for commercial purposes. Accordingly, the selected seeds of Malaysian tropical fruits were tested in this study for total phenolic content (TPC), free radical scavenging activity by 1, 1- diphenyl-2-picrylhydrazyl (DPPH) assay and metal ion chelating effect by ferrous ion chelating (FIC) assay. Extraction of antioxidant compounds from sample was done with 70% ethanol. TPCs of the seeds were expressed as gallic acid equivalents (GAE) in mg per 100 g fresh seed weight. TPC assay showed that mango seeds had the highest TPC (i.e. 32 ± 0.001 mg GAE) followed by guava seeds (i.e. 20 ± 0.001 mg GAE) and papaya seeds (8 ± 0.003 mg GAE). For DPPH assay, IC_{50} data showed that mango seed extract scavenged 50% DPPH radicals at the lowest concentration (0.11 ± 0.01 mg / mL) followed by the positive control BHA (0.13 ± 0.01 mg / mL), guava seed extract (0.26 ± 0.01 mg / mL) and papaya seed extract (0.34 ± 0.01 mg / mL). Interestingly, all seed extracts showed higher free radical scavenging activities than BHA after sample concentration of 0.60 mg / mL. However, FIC assay indicated that metal ion chelating effects of all seed extracts were weaker than BHA suggesting that the fruit seeds are not sources of good metal ion chelators. Overall, present results suggest that TPC of the seeds show strong negative correlation with their primary antioxidant activity (r= -0.985, R^2= 0.970), and not all compounds in extracts which could scavenge DPPH radicals are good metal ion chelators. Mango seeds relatively showed the highest antioxidant level and primary antioxidant activity followed by guava seeds and papaya seeds.

Keywords: Antioxidant levels, Malaysian tropical fruits, seeds

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INTRODUCTION

Vegetables and fruits have been used as natural materials to maintain human health as they may help to reduce the risk of many age-related degenerative diseases (Amin & Tan, 2002; John & James, 2005; Lee *et al*., 2007). In fact, fruits contain many antioxidant compounds such as phenolics, betalains and carotenoids. Antioxidants can be defined as substances able to inhibit or delay the oxidative damage of protein, nucleic acid and lipid caused by dramatic increase of reactive oxygen species (ROS) during environmental stress (Lim, Lim & Tee, 2006). Antioxidants act by one or more of the following mechanisms: reducing free radical activity, scavenging free radicals, potential complexing of pro-oxidant metals and quenching of singlet oxygen (Tachakittirungrod, Okonogi & Chowwanapoonpohn, 2006). Antioxidants can be classified into primary and secondary antioxidants due to their protective properties at different stages of the oxidation process. Primary antioxidants stop or delay oxidation by donating hydrogen atoms or electrons to free radicals to convert themselves to more stable products. As for secondary antioxidants, they function by many mechanisms, including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen (Maisuthisakul, Suttajit & Pongsawatmanit, 2005).

Until recently, the use of fruit seeds for commercial purposes especially as antioxidants remains low due to their lack of popularity and lack of research. In the food processing industry, edible portions of fruits are processed into products such as puree, canned slices, juice and pickles. However, most of the time the seeds will be discarded as waste since it is not currently utilised for any commercial purposes (Ajila *et al*., 2007). According to Baydar, Özkan, and Yasar (2006), many byproducts including fruit wastes contain polyphenols with potential application as food antioxidants and preventive agents against some diseases. Soong & Barlow (2004) mentioned that fruit seeds demonstrate significantly higher total antioxidant capacity and phenolic content than the edible portion. The statement was further supported by Okonogi *et al.* (2007) who mention that the peel and seed fractions of some fruits have higher antioxidant activity than the pulp fraction. Besides, polyphenolic constituents of various legume seeds have been reported to contain potential medicinal properties, including antioxidant activities (Siddhuraju & Becker, 2006). Seeds of tamarind, canola, sesame, evening primrose, flaxseed, lupinus, buckwheat, sunflower and *Rosa rubiginosa* have also been reported to be sources of antioxidants (Andrés *et al*., 2000). Needless to say, such studies directly show the high potential of seeds for use as sources of commercial antioxidants.

In this study, seeds of mango (*Mangifera indica* L.), guava (*Psidium guajava* L.) and papaya (*Carica papaya* L.) were examined for their antioxidant levels and activities. The selected fruits are among popular tropical fruits in Malaysia. Mango was originally from the Indo-Malaysian region and is considered as one of the most extensively exploited fruits for food, colourant, juice, flavour and fragrance, making it a potential ingredient in functional foods and nutraceuticals. Guava, also known locally in Malaysia as *jambu batu*, is grown commercially in many home gardens. As in many other fruits and vegetables, guava is also rich in antioxidants that help to reduce the incidence of degenerative diseases (Lim *et al*., 2006). Beside Malaysia, papaya is widely grown in India, Sri Lanka, and Thailand (John & James, 2005). The ripe flesh of papaya is usually made into sauce, pickled or preserved as marmalade and jam. Papaya juice is high in vitamin A and C (i.e. ascorbic acid), and is considered as a "health food". For that reason, antioxidant levels and activities (i.e. primary and secondary) in

seeds of guava, papaya and mango were evaluated in this study in order to investigate the possibilities of utilising the selected tropical fruit seeds as a commercial functional food or a source of nutraceuticals in the future.

MATERIALS AND METHODS

Fruit seeds

Three types of Malaysian tropical fruits were used as model system in this study namely guava (*Psidium guajava*), papaya (*Carica papaya* L.) and mango (*Mangifera indica* L.). The fruits were purchased from local fruit markets and supermarkets in Kuantan, Pahang Darul Makmur, Malaysia. The fruit samples were randomly selected off the shelves based on their colour, size and freshness. However, there is the possibility that a number of samples were imported from other countries.

Sample preparation and extraction

The fruit seeds were separated from the peels and pulps, washed and dried in the oven at 60°C for a few days. Possible detrimental effects on the total phenolic compounds of the seeds due to the drying process were taken into account (Nurliyana *et al*., in press). After the drying process, all seeds were ground into fine particle size. The powders were stored in separate screw-cap bottles at -20°C prior to extraction. For extraction, antioxidant compounds from the fruit seed powders were extracted with 70% ethanol for 8 hours utilising the Soxhlet extractor. The extracts were then filtered using Whatman No.4 filter paper and dried using rotary evaporator at 45°C for 4 hours. The dried extracts were stored at -80°C for 1 hour before they were freeze-dried. Finally, the lyophilised extracts were kept in the dark at 4°C for protection from light until further analysis.

Total phenolic content (TPC) Assay

TPCs of the seed extracts were determined according to the method by Singleton and Rossi (1965). The lyophilised seed extracts were dissolved with 70% ethanol. A volume of 0.3 mL of the ethanolic extract was mixed with 1.5 mL of Folin-Ciocalteau's reagent (diluted 10 times with distilled water) and 1.2 mL of sodium carbonate (7.5% w / v) in a test tube. Thereafter, the mixture was vortexed thoroughly, covered by parafilm and incubated in the dark for 30 min at room temperature. Then the absorbance was measured at 765 nm against a blank using Perkin Elmer Lambda 25 UV/Vis spectrophotometer. Gallic acid was used as a standard (Figure 1). The TPCs of the seed extracts were expressed as gallic acid

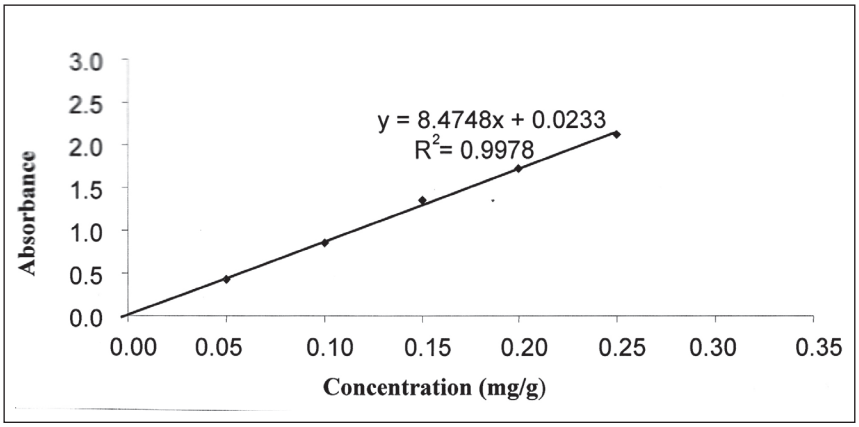

Figure 1. Concentration-response curve for gallic acid (positive control) at 765 nm

equivalents (GAE) in mg per 100 g fresh seed weight. Both samples and gallic acid were measured against 70% ethanol which was used as blank. All samples and readings were prepared and measured in triplicate.

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

Free radical scavenging activity of seed extract was measured by the method of Krings & Berger (2001). Each seed extract was prepared in a series of dilution (1.0 mg / mL, 0.8 mg / mL, 0.6 mg / mL, 0.4 mg / mL and 0.2 mg / mL) with a final volume of 10 mL. One mL of each prepared solution was then mixed with 2 mL of 0.1 mM DPPH reagent and mixed thoroughly. After 30 min of incubation at room temperature in the dark, absorbance of the mixture at 517 nm was measured using Perkin Elmer Lambda 25 UV / Vis spectrophotometer. Ethanol at 70% was used as blank**.** Standard free radical scavenger, butylated hydroxyanisole (BHA) was used as a positive control under the same assay conditions. The negative control contained 2 mL of 0.1 mM DPPH and 1 mL of 70% ethanol without seed extract or standard antioxidant. Each sample was measured in triplicate and expressed in mean average. The free radical scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_e) / A_0] \times 100\%$$

where A_0 is absorbance reading of the negative control (i.e. the blank, without extract / standard) and A_e is absorbance reading in the presence of sample. Half maximal inhibitory concentration **(**IC_{50}), the amount of sample extracted into 1 mL solution necessary to decrease by 50% the initial DPPH concentration, was derived from the percentage of radical scavenging activity (% inhibition) versus sample concentration plot (Figure 3).

Ferrous ion chelating (FIC) assay

Metal ion chelating effect of seed extract for ferrous ions Fe^{2+} was measured according to the method of Dinis, Madeira &Almeidam (1994). A 10 mL series of dilution was prepared for each seed extract at five different concentrations (0.1 mg / mL, 0.08 mg / mL, 0.06 mg / mL, 0.04 mg / mL and 0.02 mg / mL). A volume of 1.6 mL of 70% ethanol and 50 µL of $FeCl_2$ (2 mM, dissolved in distilled water) were added to 500 µL of seed extract (0.02-0.10 mg / mL). The mixtures were then mixed thoroughly and incubated for 5 min. Then, 100 µL of ferrozine (5 mM, dissolved in 70% ethanol) was added, mixed and left to incubate in the dark at room temperature for another 5 min. Ferrozine reacts with the divalent iron to form stable red or purple complex species that are very soluble in water. The absorbance of the Fe^{2+}–Ferrozine complex was then measured at 562 nm. Both ethylenediaminetetraacetic acid (EDTA) and BHA were used as positive controls. The reaction mixture containing $FeCl_2$, 70% ethanol and ferrozine without the extract, was used as the negative control. The metal ion chelating activity of each extract for Fe^{2+} was calculated as:

$$\text{Chelating effect (\%)} = [(A_0 - A_e) / A_0] \times 100\%$$

where A_0 is absorbance reading of the negative control (blank, without extract / standard) and A_e is absorbance reading in the presence of sample.

Statistical analysis

All experiments were performed in triplicate. The results are presented as in mean ± standard deviation. As for the data and graphs, they were subjected to analyses using Microsoft® Office Excel 2003.

RESULTS

TPC assay

The results showed that TPCs of the seeds ranged from 8 mg GAE to 32 mg GAE (Figure

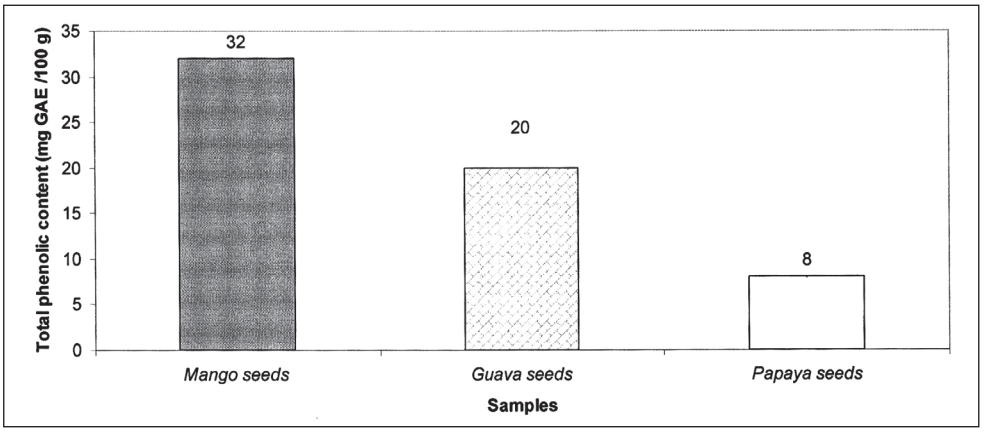

Figure 2. Level of total phenolic content per 100 g fresh seed weight in each sample

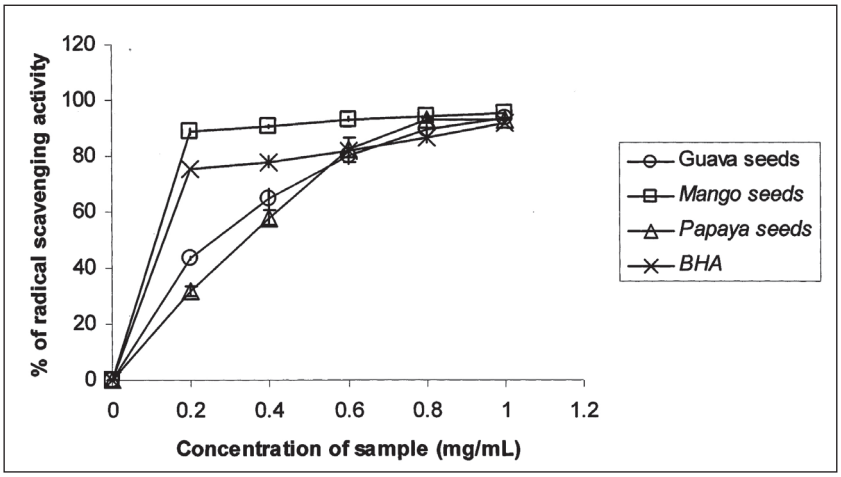

Figure 3. Comparison of free radical scavenging activity between the positive control (BHA) and samples. IC_{50} value (in mg / mL) for each sample was derived from the graph at 50% free radical scavenging activity

2). The highest TPC was shown by mango seeds (32 ±0.001 mg GAE), followed by guava seeds (20 ±0.001 mg GAE) and papaya seeds (8 ±0.003 mg GAE).

DPPH assay

Figure 3 clearly illustrates that mango seed extract had higher free radical scavenging activity as compared to the positive control, BHA over the increasing sample concentrations. Interestingly, all seed extracts showed higher free radical scavenging activity than BHA starting at sample concentration of approximately 0.6 mg / mL even if some detrimental effects on the total phenolic compounds were expected due to the drying process in the oven at 60°C for a few days. At the highest concentration of 1.0 mg / mL, mango seed extract showed the highest percentage of free radical scavenging activity (i.e. 95.12 ± 1.55%) followed by guava seed extract (i.e., 93.59 ± 2.44%), papaya seed extract (i.e. 93.19 ± 0.25%) and BHA (i.e. 91.69 ± 0.49%). In terms of IC_{50}, the lowest value was shown by mango seed extract (0.11 ± 0.01 mg / mL) followed

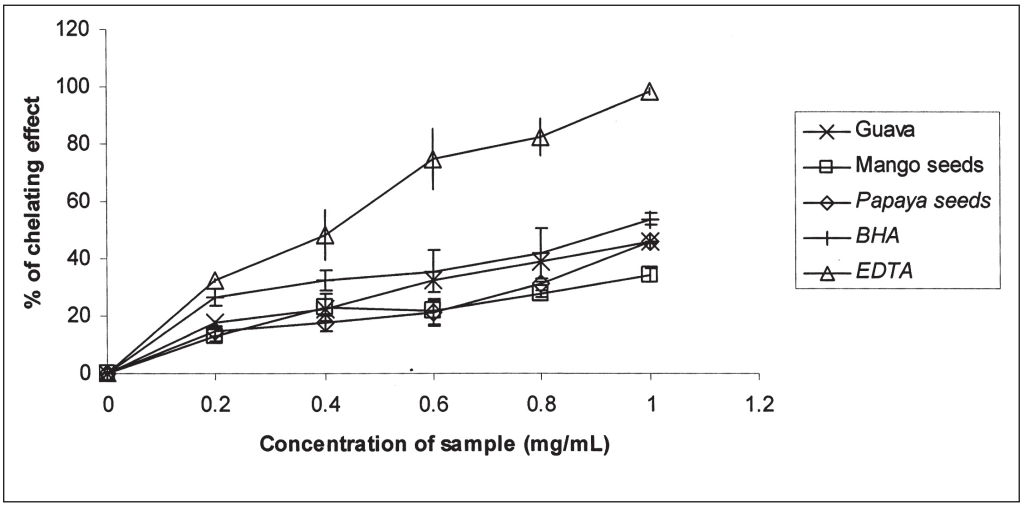

Figure 4. Comparison of ferrous ion chelating effects between EDTA, BHA and samples

by BHA (0.13 ± 0.01 mg / mL), guava seed extract (0.26 ± 0.01 mg / mL) and papaya seed extract (0.34 ± 0.01 mg / mL).

FIC assay

For the FIC assay, it was demonstrated that only reaction solutions with EDTA as the standard control showed colour change from purple to yellow as the concentration increased. The other standard control, BHA and all seed extracts did not show any obvious colour changes, although there were decreases in absorbance readings. According to Figure 4, there were no large differences between metal ion chelating effects of the fruit seed extracts. The highest metal ion chelating effect among the seed extracts was shown by guava seed extract, followed by papaya seed extract and mango seed extract. All seed extracts showed lower metal ion chelating effect than BHA.

DISCUSSION

Antioxidant level

Phenolic compounds such as polyphenols have greatly drawn the attention of food and medical scientists because of their strong *in vitro* and *in vivo* antioxidant activities and their abilities to scavenge free radicals, break radical chain reaction and chelate metals (Liu & Yao, 2006). All plant phenolic classes have the structural requirements of free radical scavengers and have potential as food antioxidants (Jayathilakan *et al*., 2007). In this study, TPCs of mango seeds, guava seeds and papaya seeds were expressed in gallic acid equivalent (Lim *et al*., 2006) since gallic acid is one of the major polyphenolic compounds that occurs in plants. Plant extracts containing high levels of gallic acid or any compound equivalent to that may be able to scavenge excessive free radicals such as superoxide anion radicals and peroxy radicals in the human body and protect human cells or tissues against oxidative stress (Rangka-dilok *et al*., 2006).

The basic mechanism of the Folin-Ciocalteau assay is an oxidation / reduction reaction based on the redox properties of antioxidant compounds that can react with the Folin-Ciocalteau reagent enhancing the measurement of phenolic concentration (Verzelloni, Tagliazucchi &Conte, 2007). In this study, a blue-coloured solution was produced when the seed extracts reacted with Folin-Ciocalteau reagent and sodium carbonate. The observation indicated the presence of phenolic compounds in all the seed extracts. The blue colour of phospho-

molybdic-phosphotungstic-phenol complex became more concentrated as the TPCs of the seed extracts increased. The present results suggest that mango seeds contained the highest TPC as compared to seeds of guava and papaya, since its extract produced the most concentrated blue solution. Phenols form the blue-coloured phosphomolybdic-phosphotungstic-phenol complex in alkaline solution. Besides, phenolics have been suggested to be the most active substances in seed extracts (Céspedes *et al*., 2008).

Primary antioxidant activity

The DPPH method was selected for measuring the primary antioxidant activity of the seed extracts because it is one of the most effective methods for evaluating the concentration of radical-scavenging materials actively by a chain-breaking mechanism (Maisuthisakul *et al*., 2005). The reduction capability of DPPH· is determined by the decrease in its absorbance at 517 nm induced by antioxidants (Liu & Yao, 2006). The decolourisation of the purple reaction solution is stoichiometric with respect to number of free radical electrons captured (Khamsah, Akowah & Zhari, 2006; Prakash *et al*., 2006).

In this study, all seed extracts showed 93-96% free radical scavenging activities at a concentration of 1.0 mg / mL suggesting their high potential as good free radical scavengers. Besides, the IC_{50} data showed that mango seed extract scavenged 50% DPPH radicals at the lowest sample concentration (i.e. 0.11 ± 0.01 mg / mL) followed by guava seed extract and papaya seed extract. Phenolic compounds generally exhibited significant scavenging effects against the DPPH free radical (Michael *et al*., 2002). Interestingly, the IC_{50} of mango seed extract was lower although not statistically significant than the positive control BHA (0.13 ±0.01 mg / mL), showing its promising potential to be exploited as commercial primary antioxidant in the nutraceutical industry. Mango seeds also showed the highest TPC as compared to guava seeds and papaya seeds. Yen, Duh & Su (2004) earlier reported that extracts with high amounts of TPC also showed a high antioxidant activity.

On the other hand, papaya seed extract which contained the lowest TPC also exhibited a high percentage of free radical scavenging activity. Khamsah *et al.* (2006) suggested that free radical scavenging activity is not due to the phenolics only based on their study on antioxidant activity of methanolic extract of *Orthosiphon stamineus*. Since all seed extracts had shown ability as high potential free radical scavengers, they can be classified as good primary antioxidants.

Secondary antioxidant activity

Iron, a transition metal, can catalyse production of toxic free radicals from peroxides by the Fenton reaction and it is implicated in many diseases. As reported earlier by Zhao *et al*. (2007), transition metal ions such as iron and copper are important catalysts for the generation of the first few radicals to initiate the radical chain reaction or the radical mediated lipid peroxidation. Metal ion chelating capacity is significant since they reduce the concentration of catalysing transition metal in lipid peroxidation. Chelating properties may be attributed to endogenous chelating agents, mainly phenolics. Some phenolic compounds have properly oriented functional groups that can chelate metal ions (Manian *et al*, 2007). It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised form of the metal ion (Oktay, Gulcin & Kufrevioglu, 2003).

In order to evaluate the potential of mango seeds, guava seeds and papaya seeds as secondary antioxidants, metal ion chelating activity of each extract was tested

against ferrous ion (Fe^{2+}). The metal ion chelating ability of an extract measures how effectively the compounds in it can compete with ferrozine for ferrous ion. Ferrozine can quantitatively form complexes with Fe^{2+}. The presence of antioxidant compounds in seed extracts can disrupt the formation of ferrozine- Fe^{2+} complex resulting in decolourisation of red or purple colour of the complex (Velavan *et al.*, 2007). Measurement of colour reduction, therefore, allowed the estimation of the metal ion chelating activity (Kosem, Han & Moongkarndi, 2007). In this study, the purple colour of reaction solutions did not change much in the presence of all seed extracts, and their percentages of metal ion chelating effect were lower than the standard control BHA and approximately two times less than EDTA. EDTA is a strong metal chelator (Gülçin, Berasvili & Gepdiremen, 2005), hence, it was used as standard metal chelator agent in this study. The results suggest that compounds in seeds of mango, guava and papaya can not completely obstruct the generation of ·OH radicals from the Fenton reaction indicating weak metal ion chelating effects.

Correlation between results of TPC, DPPH assay and FIC assay

In this study, the results suggest a strong negative correlation between the TPC and the free radical scavenging activity ($r = -0.9849$, $R^2 = 0.9700$). According to Prior, Wu & Schaich (2005), the Folin-Ciocalteau assay gives a crude estimate of the total phenolic compounds present in an extract, whereby the free radical scavenging assay is not specific to polyphenols but many interfering compounds may react with the reagent. Various phenolic compounds respond differently in DPPH assay, depending on the number of phenolic groups they have (Singleton & Rossi, 1965). The responses of phenolics for antioxidant activity estimated by various methods also depend on their chemical structures (Zhao *et al.*, 2007). Furthermore, Tawaha *et al.* (2007) suggest that the negative correlation between TPC and antioxidant activity may be due to the TPC that does not necessarily incorporate all the antioxidants that may be present in an extract, for instance betalain that contains both phenolic and non-phenolic structures. Hence, this may explain the negative correlation specifically between the TPC and the free radical scavenging activity observed in this study.

The present results also suggest that not all compounds in the seed extracts which could scavenge DPPH radical are good chelators of Fe^{2+} ion. The metal ion chelating activity of seed extracts might partly depend on the functional groups and content of individual functional groups in seed extracts (Zhao *et al.*, 2007). Therefore, antioxidant activity of the seed extracts could not be predicted on the basis of its TPC only. This is due to the synergism of polyphenolic compounds with one another or with other components present in an extract, that may contribute to the overall observed antioxidant activity (Ordonez *et al.*, 2005).

CONCLUSION

Among the seed extracts of Malaysian tropical fruits analysed in this study, it was found that the highest TPC was in mango seed extract (mango seeds > guava seeds > papaya seeds). The highest free radical scavenging activity backed by IC_{50} data was also exhibited by mango seed extract (mango seeds > guava seeds > papaya seeds). In contrast, the highest percentage of metal ion chelating effect based on FIC assay was indicated by guava seed extract (guava seeds >papaya seeds> mango seeds). Furthermore, there is a strong negative correlation between the TPC and the free radical scavenging activity. The present results summarise that mango seeds relatively has the highest antioxidant level and primary antioxidant activity followed by guava seeds and papaya seeds, showing its promising potential to be

exploited as commercial primary antioxidant in the nutraceutical industry. Nonetheless, in order to gain a better view of the antioxidant levels and activities in seeds of mango, guava and papaya, further studies on purification, identification and quantification of each phenolic compound and other non-phenolic compounds are necessary in future.

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