

PHYTOCHEMICAL SCREENING AND ACTIVITIES OF HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT OF SOME FRUIT PEELS

(Kajian Fito-kimia dan Aktiviti Hidrofilik dan Lipofilik Antioksida untuk Beberapa Kulit Buah)

Khairusy Syakirah Zulkifli¹, Noriham Abdullah¹, Aminah Abdullah², Nurain Aziman¹,
Wan Saidatul Syida Wan Kamarudin¹

¹Faculty of Applied Sciences,
Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

²School of Chemicals Sciences and Food Technology,
Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

*Corresponding author:

Abstract

This study was conducted to screen the secondary metabolites compounds including alkaloids, tannins, saponins and flavonoids as well as to determine the antioxidant activities of four types of fruit peels namely *Psidium guajava* (guava), *Mangifera indica* (Chakonan mango), *Citrus sinensis* (Navel orange) and *Malus sylvestris* (Granny Smith apple). The hydrophilic and lipophilic antioxidant activities were investigated using three different assays such as Ferric Reducing Antioxidant Power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and Oxygen Radical Absorbance Capacity (ORAC). Total Phenolic Content (TPC) and Total Flavonoids Content (TFC) were also conducted and the correlations between the antioxidant assays with TPC and TFC were evaluated. The TPC in the peels extract ranged between 204.90 to 517.00 mg GAE/g extract weight while TFC ranged between 97.48 to 177.86 mg QE/g extract weight. The FRAP, EC₅₀ scavenging activity and ORAC values were 18.78 to 45.36 mM TE/100g extract weight, 0.146 to 0.717 mg/ml scavenging effect and 37.54 to 60.59 μ M TE/g extract weight respectively. The extract of *M. indica* peels appeared to be as potent as ascorbic acid with maximum inhibition of 74% at 200 ppm. *M. indica* peels showed highest value in all antioxidant assays and in TPC while the highest in TFC was found in *M. sylvestris* peels. There were strong correlations between all antioxidant assays with TPC but very weak correlations with TFC. This study suggested that the extracts of fruit peels are convenient to be use as functional ingredients in food product development as they are rich in antioxidant activities.

Keywords: Secondary metabolites, hydrophilic, lipophilic, antioxidant, fruit peels

Abstrak

Kajian ini dijalankan untuk mengkaji kompaun metabolit sekunder termasuklah alkaloid, tannin, saponin dan flavonoid dan mengkaji aktiviti antioksida keatas empat jenis kulit buah-buahan antaranya *Psidium guajava* (jambu batu), *Mangifera indica* (mangga madu), *Citrus sinensis* (oren Navel) and *Malus sylvestris* (epal Granny Smith). Aktiviti hidrofilik dan lipofilik antioksida dikaji menggunakan tiga ujian berbeza iaitu ujian penurunan ferrik (FRAP), pemerangkapan radikal bebas 2, 2-difenil-1-pikrilhidrazil (DPPH) dan kapasiti menyerap oksigen radikal (ORAC). Kandungan fenolik total (TPC) dan kandungan flavonoid total (TFC) turut dijalankan dan korelasi antara ujian antioksida dengan TPC dan TFC dinilai. TPC di dalam ekstrak kulit buah berada dalam lingkungan 204.90 hingga 517.00 mgGAE/100g berat ekstrak manakala TFC berada di antara 97.48 hingga 177.86 mgQE/100g berat ekstrak. Nilai FRAP, aktiviti pemerangkapan EC₅₀ dan nilai ORAC masing-masing ialah 18.78 hingga 45.36 μ M TE/g berat ekstrak, 0.146 hingga 0.717 mg/ml kesan pemerangkapan dan 37.54 hingga 60.59 μ M TE/g berat ekstrak. Ekstrak kulit *M. indica* menunjukkan kekuatan setaraf asid askorbik dengan perencatan semaksima 74% pemerangkapan di kepekatan 200 ppm. Ekstrak kulit *M. indica* menunjukkan nilai tertinggi di dalam semua ujian antioksida dan TPC manakala nilai TFC tertinggi dijumpai di dalam ekstrak kulit *M. sylvestris*. Korelasi yang kukuh ditunjukkan antara semua ujian antioksida dengan TPC tetapi korelasi yang lemah dengan TFC. Kajian ini menunjukkan bahawa ekstrak kulit buah-buahan tersebut sesuai digunakan sebagai bahan berfungsi di dalam penghasilan produk makanan berikutan kekayaan aktiviti antioksida.

Kata kunci: metabolit sekunder, hidrofilik, lipofilik, antioksida, kulit buah-buahan

Introduction

Free radicals are reactive molecules that initiate in human metabolism in two ways – naturally produced by body system to neutralize the presence of viruses and bacteria or by environmental factors like smokes, pollution, radiation and others. The radical chain reaction with proteins, DNA and cell membrane will give harmful result to human body [1]. Although the anti-free radical defence system like antioxidants, enzymes, vitamins and other novel minerals are naturally available, however, the potency to oppose radicals is depends on several factors including amount of antioxidant present in the body, amount of radicals and also aging factor [2].

The ongoing evolution of newer and wider plants species gain much attention among researchers for their naturally secondary metabolites especially phenolic compounds, flavonoids, alkaloids, saponins, steroid and terpenes. These metabolites own antioxidant properties because of their capability as hydrogen donator, reducing agent, free radical scavenger and binding with metal ions [3]. Among the plants variety, fruits become priority area as that they are locally abundant, low in cost and can be consumed in various occasions. Besides, they were highly recommended for daily intake due to positively correlated to the reducing of cancers and chronic diseases. Surprisingly among others, the peel was found to contain much higher antioxidant compound compared to other fruit fractions [4][5][6]. More recent work has shown the peels contain anywhere from two to six times more phenolic compounds and two to three times more flavonoids in the peels when compared to the flesh. Moreover, some substances like anthocyanin, flavonol, kaempherol and xanthone glycoside in fruits were originally invented from its peels [7][8].

Phenolic compounds are mostly polar, thus increasing solvent polarity will increased the extraction yields. Aqueous extraction were used due to their wide solubility selection and from toxicological point of view, water is much safer than other organic solvents (methanol, acetone and chloroform) [9]. In addition, boiling condition will increased the solubility of phenols and enhanced the breakdown of high molecular weight phenolics into free form which also led for the extraction of more polyphenols.

Therefore, in this study, a highly polar water extraction was used and determined for their phytochemical compounds and their antioxidant activity in (1) hydrophilic assay using DPPH scavenging activity and Ferric Reducing Antioxidant Power (FRAP) and (2) total antioxidant activity of hydrophilic and lipophilic assay using Oxygen Radical Antioxidant Capacity (ORAC) on four types of fruit peels *Psidium guajava* (guava), *Mangifera indica* (mango), *Citrus sinensis* (Navel orange) and *Malus sylvestris* (Granny Smith apple).

Materials and Methods

Raw Materials and Sample Preparation

Four types of fruits namely *Psidium guajava* (guava), *Mangifera indica* (mango), *Citrus sinensis* (Navel orange) and *Malus sylvestris* (Granny Smith apple) were obtained from local wet market at Seksyen7, Shah Alam. All the fruit were peeled, washed with tap water and dried. The dry peels were boiled in water for 10 min with 1:20 ratio. They were filtered with Whatman #1 filter paper and further concentrated by rotary vacuum evaporator at temperature 60 °C. The samples were freeze dried and kept at -20°C [10].

Preliminary Phytochemical Screening

The four extracts were evaluated for the presence of different phytochemicals using procedures of Aiyegoro and Okoh (2010) [11].

Alkaloid

The crude extract was mixed with acid alcohol (10% acetic acid in ethanol), boiled and filtered. 10% ammonia was added into the filtrate followed by chloroform. Two layers of solution were formed. The chloroform layer was extracted with 5% hydrochloric acid and few drops of Mayers reagent were added. A white or cream colour solution will be produced.

Flavonoid

The crude extract was dissolved in methanol and boiled. A small piece of magnesium ribbon was added to the mixture followed by few drops of concentrated HCl. The mixture will turn to red or orange if flavonoid were present.

Tannin

The crude extract was dissolved and heated in distilled water until boiled. Few drops of ferric chloride were added and blue-black or brownish green solution will be produced indicating the presence of tannin.

Saponin

The crude extract was diluted and boiled in distilled water. The solution was shaken vigorously until stable persistent froth was observed. (No froth = negative; froth less than 1 cm = weakly positive; froth 1.2 cm high = positive; and froth greater than 2 cm high = strongly positive).

Steroid

The crude extract was dissolved in chloroform and filtered. Concentrated sulphuric acid was carefully added to the filtrate and form layers. A reddish brown colour at the interface indicates the presence of steroid ring.

Total Phenolic Content

Capacity of total phenolic contents was determined using Singleton and Rossi (1965) [12] method with slight modification. Gallic acid was used as a standard with varied concentration from 200 ppm to 1000 ppm. Both samples and standards were reacted with Folin-Ciocalteu reagent (1:1), 7.5% (w/v) sodium carbonate and water before observed under UV-Vis spectrophotometer at 760 nm absorbance. The total phenolic content was expressed as mg gallic acid equivalents per gram extract weight (mgGAE/100g extract weight).

Total Flavonoid Content

The total flavonoid content was conducted according to Marinova et al. (2005) [13]. The extracts and standards respectively were reacted 5% NaNO₂, 10% AlCl₃ and 1M NaOH. Quercetin was used as standards with concentration varied from 200 ppm to 1000 ppm. The mixture was measured at 510 nm and expressed as mg quercetin equivalent per gram extract weight (mgQE/100g extract weight).

Ferric Reducing Antioxidant Power Assay

The FRAP was assessed according to Benzie and Strain (1996) [14] using a UV-Vis spectrophotometer at 593 nm. The method based on the reduction of the Fe³⁺-TPTZ complex into blue colour of ferrous compound at low pH. Briefly, the sample extract and standards respectively, will be mixed with 8.7 mL of working FRAP reagent and incubated in dark for 1 hour at 50°C. FRAP reagent must be always freshly prepared by mixing 10mM TPTZ solution with 20 mM FeCl₃.6H₂O and 300 mM acetate buffer (pH 3.6) with ratio 10:10:100. Calibration curve was prepared by using trolox as standard (100-500 ppm).

DPPH Free Radical Scavenging Activity

DPPH assay was conducted according to method by Heimler et al (2005) [15]. The sample extracts and standards (BHA and ascorbic acid) were prepared at various concentrations (200-1000 ppm) and mixed with ethanolic solution of DPPH with a concentration of 0.04 mg/mL. After stand for 20 min in the dark, the mixtures were measured at 517 nm against ethanol as blank using UV-Vis Spectrophotometer. The result obtained were calculated using formula:

$$\text{Scavenging Activity AA(\%)} = (\text{Abscontrol} - \text{Abssample}) / \text{Abscontrol} \times 100$$

Oxygen Radical Antioxidant Capacity

ORAC assays were carried out on a fluorescein plate reader. The temperature of the incubator was set to 37°C. Procedures were based on the method of Wu et al (2004) [16]. Three solutions were prepared. The peroxy radical generator (AAPH) was prepared by mixing AAPH with assay buffer. The Trolox standard was prepared in various concentrations by dissolving 1.5mM Trolox with assay buffer. Finally, fluorescent probe was prepared by mixing

fluorescein in assay buffer. The excitation and emission wavelength were set at 485 nm and 528 nm. 25µl of diluted sample, blank, and Trolox calibration standard were transferred to 96-well plates and mixed with 150 µL of fluorescein solution and incubated for 5min at 37°C. 25 µL of AAPH solution were lastly injected. The fluorescence was measured every minute for 30 minutes. The final ORAC values were calculated using the net area under the decay curves and were expressed as micromolar Trolox equivalents (TE) per 100 g of extract weight. The area under curve (AUC) was calculated as

$$AUC = 0.5 + \sum (\int_0/\int_0 + \int_1/\int_0 + \int_2/\int_0 + \int_{30}/\int_0) + (0.5 \times \int_{30}/\int_0)$$

Statistical Analysis

All the results were expressed as mean values ± standard deviation. Comparisons will be performed by analysis of variance (ANOVA). Statistical analyses will be run using SPSS V. 6.0 software. The correlations among the data were calculated using Pearson's correlation coefficient (r) and P<0.05 was considered significantly different.

Results and Discussion

Screening of Secondary Metabolites

The screening of the biological activity of secondary metabolites was done in order to discover new therapeutic agents as well as an adjunct to medicinal knowledge. Secondary metabolites were known for their ability as antioxidant, antifungal, anti-inflammatory and antimicrobial properties. The screening was carried out on the crude extract of *Psidium guajava*, *Mangifera indica*, *Malus sylvestris* and *Citrus sinensis* peels which shown the presence of several phytochemical compounds as shown in Table 1.

From the findings, alkaloids are present in all extracts except *M. indica*. Flavonoids were present in all the extracts while saponins were only present in *P. guajava* and *M. sylvestris*. Steroids and tannins were present in all extracts except *M. indica* and *M. sylvestris* extracts respectively.

Alkaloids consist of heterocyclic nitrogen which was synthesized from amino acids or their immediate derivatives in plant cells [17]. They are insoluble in water therefore had to be dissolved in aqueous acid solution and extracted using organic solvent in basic medium. Although alkaloids are abundantly present, in all plants but usually they are localized in one part. Some parts may not contain alkaloids because of their toxicity properties and they provide bitter taste to food.

Flavonoids are natural antioxidant that present in virtually all plant cells. They are polar compounds and quite acidic, therefore, a dilution with methanol and concentrated hydrochloric acids are required to cleave the flavonoids. Saponins are naturally occurring glycosides in plants consisting of hydrophilic sugar that attached to lipophilic triterpene or steroid triterpene which determine the polarity and hydrophobicity of the compounds [18]. The ability of a saponin to foam is caused by the combination of the nonpolar sapogenin and the water-soluble side chain. Tannins are also parts of phenolic, can be derived into two forms; water soluble of yellow-brown hydrolysable tannins and condensed tannins. Tannins were hydrolyzed when reacted with dilute acid to which blue, blue-black, green or blue-green colour solution were produced indicating the presence of tannins.

Table 1. The secondary metabolites presence in selected fruit peels

Phytochemicals	<i>Psidium guajava</i>	<i>Mangifera indica</i>	<i>Malus sylvestris</i>	<i>Citrus sinensis</i>
Alkaloid	+	-	+	++
Flavonoid	+	+	+	+
Saponin	+	-	+	-
Steroid/ Terpenoid	+	-	+	+++
Tannin	+	+++	-	++
Phlobatannin	-	-	-	-

+ = Present; - = Absent.

Total Phenolic Content

The total phenolic content of the extracts were determined using Folin-Ciocalteu (FC) colorimetric method. In this method, the yellow molybdotungstophosphoric heteropolyanion reagent with 6⁺ oxidation state will react with the phenols present in the extract. The phenols will reduced the reagent through electron transfer reaction thus resulting for the formation of 5⁺ oxidation state of molybdotungstophosphate. The mixture will turns blue in alkaline medium that provided by sodium carbonate [19].

The total phenolic contents are shown in Figure 1. The maximum polyphenol content was recorded in *M. indica* peel extract with 517±21.17 mg GAE/g extract weight and the lowest was recorded in *M. sylvestris* peel extract with 204.9±13.76 mg GAE/g extract weight. However, the lower value of TPC was observed by other researchers [20] [6]. The different levels of phenolic content not only depend on the position and numbers of particular hydroxyl groups on its aromatic ring [21], but also because of different plants tissues, cultivars, and different extraction methods [22] [23]. Despite from that, the FC reagent was also non-specific on phenol compounds as they are able to react with any reducing substances like sugars [24].

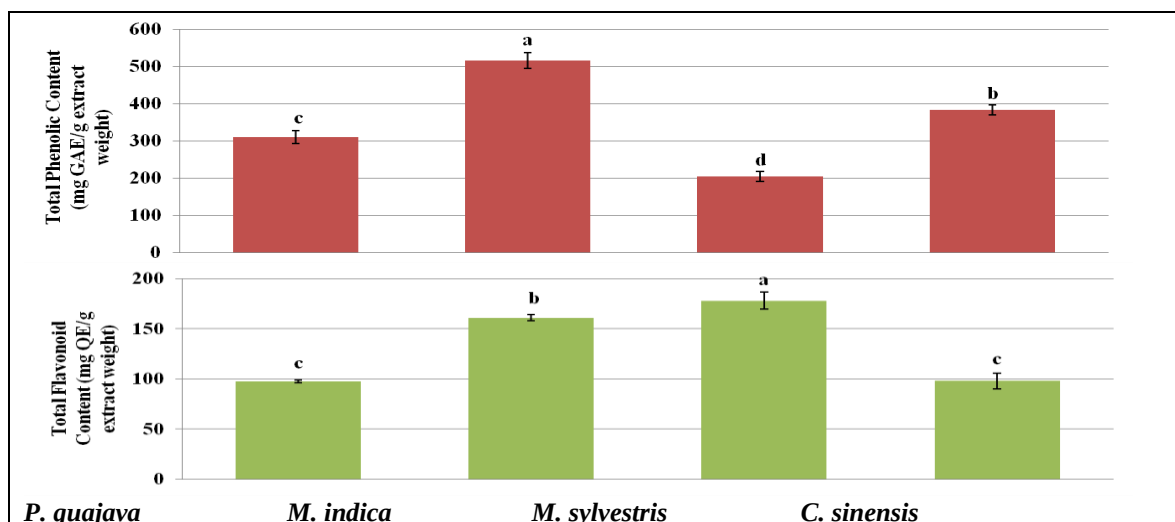


Figure 1. The total phenolic and total flavonoid content were expressed as gallic acid equivalent (GAE) and quercetin equivalent (QE) per gram extract weight respectively. The values were expressed as mean $\pm$ standard deviation ($n=5$) and the significant difference at the level $p < 0.05$

Total Flavonoid Content

The procedure is based on the principal that, aluminium ion from aluminum chloride (AlCl_3) will interacts with the ketone and hydroxyl groups of flavonoids in sample extracts through electron transfer reaction at high pH that caused precipitation into yellow colour [25]. According to Chang et al. (2002) [26] the AlCl_3 method able to determine mainly the flavones and flavonols groups, while 2, 4-dinitro phenylhydrazine calorimetric method is use to determine the flavanone groups. However, as flavonols are able to exhibit higher antioxidant compared to flavones and flavanones, therefore this method is much preferable. Yu et al. (2005) [27] claimed that a small quantity of either flavonols or flavones will possess higher antioxidant properties than abundance of flavanone.

Compared to the total phenolic content, the *M. sylvestris* peels show an opposite character for total flavonoid content (Figure 1). The *M. sylvestris* peels contain the highest flavonoid content with 177.86±8.51 mg QE/g extract weight followed by *M. indica* peels with 160.92±3.16 mg QE/g extract weight. Previous researchers had identified

the presents of triterpenoids, quercetin and xanthone glycosides in both peels [28] [29]. The total flavonoid content in *C. sinensis* and *P. guajava* peels have no significant different. According to Bocco et al. (1998) [30], citrus peels are very high in flavanones instead of flavones or flavonols which responsible for the bitter taste and the bright colour of orange.

DPPH Radical Scavenging Activity

DPPH assay is used to determine the scavenging potential of the antioxidant extract based on its capability as hydrogen donator. DPPH gives a strong absorption band at 517nm in visible region. When the phenolic compounds in the extract react with the stable DPPH radical, the absorption reduced and DPPH is decolourised from blue complex into light yellow [20]. This discolouration is depends on the intrinsic concentration of present antioxidant and its reactions speed towards DPPH. The degree of reduction in absorbance measurement is indicative of the radical scavenging power of the extract.

The scavenging activities of selected fruit peels and standards at various concentrations are shown in Figure 2. *M. indica* peel shows a very significant scavenging ability compared to other peels. The crude extract of *M. indica* peel appeared to be as potent as ascorbic acid with a maximum inhibition of 74% at 200 ppm which is comparable to 96% inhibition for ascorbic acid at the same concentration. Besides, the EC_{50} value for *M. indica* peel (Table 1) had the lowest value whereas *M. sylvestris* peel had the highest EC_{50} .

EC_{50} value is used to determine the concentration of antioxidant required to scavenge 50% of DPPH radicals. According to Maisuthisakul et al. (2007) [31], the smaller value of EC_{50} corresponds to a higher antioxidant activity of the plant extract. Balsundram et al. (2005) [32] claimed that the radical scavenging activities of various phenolic compounds respond differently depending on the number and location of hydroxyl groups of phenolics. In addition, the solubility of the phenolics in the test medium may also significantly affect their ability to act as antioxidants.

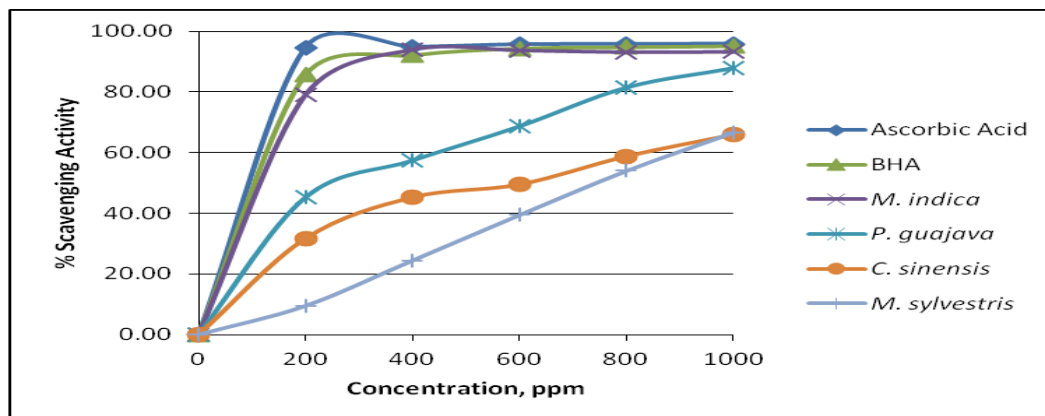


Figure 2. Scavenging activities of selected fruit peels and standards towards DPPH radicals. The values were expressed as mean $\pm$ standard deviation ($n=5$). BHA and ascorbic acid were used as the standards.

Table 2. Ferric Reducing Antioxidant Power (FRAP), DPPH radical scavenging activity (EC₅₀) and ORAC value of the selected fruit peels.

TYPES	EC ₅₀ ^A (mg/ml)	FRAP ^B (mM/100g)	ORAC ^C (μM TE/g)
<i>Psidium guajava</i>	0.2652	27.30 ± 0.573 ^c	54.91 ± 8.28 ^b
<i>Mangifera indica</i>	0.1461	45.36 ± 0.582 ^a	59.83 ± 2.342 ^a
<i>Malus sylvestris</i>	0.7165	18.78 ± 0.086 ^d	37.54 ± 6.467 ^c
<i>Citrus sinensis</i>	0.5157	34.65 ± 1.497 ^b	60.59 ± 3.454 ^a

^AEC₅₀ data expressed as milligram per millimeter mg/ml). ^BFRAP data expressed as milimolar per 100 gram (mM/100g) ^CORAC data expressed as micromoles of Trolox equivalents per gram (μmol of TE/g).

Ferric Reducing Antioxidant Power Assay

The reducing antioxidant power of sample extract is depends on its electron transfer ability towards the FRAP reagent. In this hydrophilic assay, the ferric tripyridyl triazine salt will be reduced into blue colour of ferrous ion at low pH medium [33].

The results shown in Table 2 are different than other referred journals that using the same assay method. Guo et al (2003) [34] estimated FRAP value for *P. guajava* peels was about 10.24±0.24 mM/100g, *M. indica* peels (10.13±0.37 mM/100g) and *C. sinensis* peels (5.69 ± 0.26 mM/100g). 7.28±0.09 to 12±0.24 mM/100g reducing activity was possessed by *M. sylvestris* peels through the test carried out by Valavanidis et al. (2009) [23]. The differences might be caused by different extraction method and different plant origin. The resulting Fe(II) is a pro-oxidant that able to generate additional radicals and the low pH medium (pH 3.6) will lead to protein precipitation.

Oxygen Radical Antioxidant Capacity (ORAC)

ORAC is one of the latest assay methods with lots of improvement for determining antioxidant activities possessed by both hydrophilic (phenolics and flavonoids) and lipophilic (carotenoids) antioxidant. ORAC is different than other assay as there are free radical or oxidants that been applied [35]. The oxidative degradation occurred through the spontaneous decomposition of AAPH and produces peroxy radicals. The radicals will damage the fluorescent molecules resulting in the loss of fluorescence. ORAC measures the inhibition of free radical damage by protection of antioxidant against the change of probe fluorescence through hydrogen transfer reaction. Results in Table 2 shows that *C. sinensis* had the highest ORAC value while *M. sylvestris* had the lowest.

Relations between Antioxidant Assays with Total Phenolic Content and Total Flavonoid Content

It is extremely important to point out that there is a positive correlation between antioxidant activity with the total phenolic and total flavonoids content of the extracts. The correlations in Table 3 show weak correlations between the antioxidant assays (FRAP, DPPH and ORAC) towards the total flavonoid content but strong correlations for total phenolic content.

The strong correlations show that phenols are very important plant constituents that contributed for antioxidant activity. This mainly due to the present of hydroxyl groups which were known for their capability as good electron acceptor. In most referred journals claimed that flavonoids, which the main classes in the phenolic groups, are the main contributor in antioxidant activities. However the weak correlations show that the antioxidant activity in the sample does not only contributed by flavonoids but they are widely distributed among the phenolics groups like B-carotene, ascorbic acid and anthocyanins [22] as well as from other secondary metabolites such as alkaloids and saponins.

Table 3. The correlation between Total Phenolic and Total Flavonoid Content towards different antioxidant assays (EC₅₀, FRAP and ORAC).

	EC ₅₀	FRAP	ORAC
TPC	0.621	0.998	0.667
TFC	0.059	0.423	0.009

Saponins have unique residue like 2, 3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran- 4-one (DDMP), which allows saponins to scavenge superoxides by forming hydroperoxide intermediates which prevent bio-molecular damage. Meanwhile, alkaloids also contribute in inhibiting lipid peroxidation by attenuating oxidative damage of hyaluronic acid and cartilage collagen. These phytochemicals act synergistically in mechanism which the easily oxidized compounds will be regenerated by less active [36].

Conclusion

From the present data, the antioxidant activity assays showed that *M. indica* peels are the strongest antioxidant agent followed by *C. Sinensis* peels, *P. guajava* peels and lastly *M. sylvestris* peels. The high antioxidant properties of *M. indica* may be caused by the high level of Total Phenolic Content which showed strong correlation with antioxidant assays as well as the abundance of tannins present in the peel extract. Fruit peels can be an alternative use in pharmaceuticals and food industries.

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