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Assessment on the Antioxidant and Antibacterial Activities of Selected Fruit Peels

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Abstract: Four fruits namely *Psidium guajava* (seed guava), *Mangifera indica* var. Chakonan (Chakonan mango), *Citrus sinensis* var. Navel (Navel orange) and *Malus sylvestris* (Granny Smith apple) were analysed before the peels were studied for their total phenolic content (TPC), total flavonoid content (TFC), *in-vitro* antioxidant activities using four assays including Ferric Reducing Antioxidant Power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, Beta-carotene bleaching and Oxygen Radical Absorbance Capacity (ORAC) and their antibacterial activity using disc diffusion method against six bacteria. *M. indica* extract showed highest in TPC and TFC with 480.11 mg GAE/g and 50.37 mgQE/g respectively. In addition, *M. indica* peels also showed high antioxidant activity in most assays other than showing potent antibacterial inhibitor against both Gram-positive and Gram-negative bacteria. Overall, this study provides supportive evidence for the superiority of *M. indica* peels as an excellent source of bioactive compounds and the potential of fruit by-product to be utilised as functional food ingredients and nutraceutical products.
Keywords: *fruit peels, antibacterial, biological activities, in-vitro antioxidant.*

Introduction

On the basis of the research attempt for the past two decades, polyphenols as plants natural free radical defence were acknowledged to be beneficial to human health as antioxidant, antibacterial, and anti-diabetic agent. The importance of natural bioactive compounds has led to the development of a large and potential market for natural sources in food products and pharmaceutical. Fruits by-products such as seeds, peels, stems, barks and leaves usually been discarded and currently give a serious disposal problem in food and agricultural industries. Thereby, extensive researches on utilizing these waste fractions have been carried out worldwide. As the outcome, the peel was found to contain much higher beneficial compounds that possessed antioxidant capacities compared to other fruit fractions including the flesh itself in accordance with previous studies [1, 2, 3].

The natural bioactive compounds in fruits such as carotenoids, quercetin derivatives, phenolic acids

and anthocyanins were found originally in the peels and the concentration decreased towards the flesh. Recent studies also confirmed the substantially higher amount of phenolic compounds and ascorbic acids in the peel than in the pulp for most of the fruits [4,5]. Jeong *et al*. (2004) [6] claimed that the skin usually contain higher bioactive compounds in order to protect the inner materials from insects and microorganisms deterioration.

The aim of this study is to highlight some biological activities of aqueous extracts of selected fruit peels. In essence, we quantify the phenolic content, determined *in vitro* antioxidant activities and their potential bacterial inhibitor. The selected fruits including a local cultivar of *Psidium guajava* (seed guava) and *Mangifera indica* var. Chakonan (Chakonan mango) together with two imported fruits, *Citrus sinensis* var. Navel (Navel orange) and *Malus sylvestris* (Granny Smith apple).

Materials and methods

Sample preparation

Fresh fruits with no apparent physical or microbial damage were obtained from local market in Shah Alam, Selangor, Malaysia. Samples were local fruits of *Psidium guajava* (guava) and *Mangifera indica* var. Chakonan (Chakonan mango) as well as imported fruits of *Citrus sinensis* var. Navel (Navel orange) and *Malus sylvestris* (Granny Smith apple). All of the fruits were of eating quality, and they were selected based on shape, size and weight uniformity and external colour at commercial maturity stage. All of the fruits were washed with tap water, weighed and studied for physico-chemical characteristics before they were peeled. The pulp of each fruits was studied for the pH, soluble solids and titratable acidity to determine their maturity indices.

Extraction Procedure

The extraction procedure was carried out according to Duh *et al*. (1999) [7]. 100 g of each fresh peel were dried in cabinet drier overnight at 60 ºC. The dried peels were then blended using a blender before boiled in water for 10 minutes with 1:20 peel to water ratio. They were re-blended until homogenized before filtered with Whatman No. 1 filter paper. The supernatants were further concentrated at 60ºC by rotary vacuum evaporator (Buchi distillation R-210, Switzerland). The samples were lyophilised using freeze drier (Christ Alpha 1-4 LD Plus, Germany) and kept at -20ºC until further use. Light exposure was avoided throughout the extraction process by covered each apparatus using aluminium foil.

Determination of Total Phenolic Content (TPC)

Gallic acid was used as a standard with varied concentration from 100 ppm to 500 ppm. Both samples and standards were mixed with Folin-Ciocalteu reagent (1:1), 7.5% (w/v) sodium carbonate and diluted with water before observed under UV-Vis spectrophotometer at 760 nm absorbance. Each measurement was repeated five times and TPC was expressed as mg Gallic acid equivalent per gram extract weight (mg GAE/g extract weight) [8].

Determination of Total Flavonoid Content (TFC)

The extracts and standards respectively were reacted with 5% $NaNO_2$, 10% $AlCl_3$ and 1 M NaOH. Quercetin was used as standards with concentration varied from 200ppm to 1000ppm. The mixture was measured at 510 nm using UV-Vis spectrophotometer. Each measurement was repeated five times and TFC was expressed as mg Quercetin equivalent per gram extract weight (mg QE/g extract weight) [9].

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP was assessed according to Benzie and Strain (1996)[10], using a UV-Vis spectrophotometer at 593 nm. The method is based on the reduction of the Fe^{3+}-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 an hour at 50°C before analyzed. FRAP reagent must be freshly prepared by mixing 10 mM TPTZ solution with 20 mM $FeCl_3.6H_20$ and 300 mM acetate buffer (pH 3.6) with ratio 10:10:100 and were incubated at 37°C for 10 minutes. Calibration curve was prepared by using trolox as standard (100-500 ppm). The results were expressed as millimolar per 100 gram extract weight (mM/100g).

DPPH Free Radical Scavenging Activity Assay

The sample extracts and standards (butylated hydroxylanisole 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 standing for 20 min in the dark, the mixtures were measured at 517 nm against ethanol as blank using UV-Vis Spectrophotometer[11]. Each measurement was repeated five times. The percentage of remaining DPPH against the sample and standard concentration was plotted to obtain the amount of antioxidant necessary to decrease the initial

concentration of DPPH by 50% (EC_{50}) using this formula:

Scavenging Activity AA(%) = ($Abs_{control}$ – Abs_{sample})/ $Abs_{control}$ x 100

Based on the parameter EC_{50}, the result was expressed in terms of µg extract per gram standard equivalent DPPH in the reaction medium.

Beta-carotene Bleaching Assay

Beta-carotene solution (0.002 g in 10 ml chloroform) was mixed with 60 µl of linoleic acid and 600 µl of Tween 20. This mixture was then evaporated at 40°C for 10 min by using a rotary vacuum evaporator to remove chloroform and immediately diluted with 300 ml of distilled water. The mixture was shaken vigorously to form an emulsion. About 5 ml of the emulsion were transferred into test tubes each containing 200 µl of extracts and standards. Standard butylated hydroxytoluene (BHT) and butylated hydroxylanisole (BHA) in methanol, at the same concentration as samples, were used as reference. The tubes were then gently shaken and placed at 50°C in a water bath for 2 h. The absorbance of the extracts, standard and control were measured at 470 nm, using a spectrophotometer against a blank consisting of an emulsion without Beta-carotene [12]. The measurements were carried out at initial time (t=0) and at final time (t=2). All samples were assayed in duplicates.

Oxygen Radical Absorbance Capacity (ORAC) Assay

ORAC assay were carried out on a fluorescein plate reader. The temperature of the incubator was set to 37°C. Three solutions were prepared. The peroxyl radical generator (AAPH) was prepared by mixing 175 mg of AAPH with 2.7 ml assay buffer. The Trolox standard was prepared in various concentrations by dissolving 20 µl of 1.5 mM Trolox with 280 µl assay buffer. Finally, fluorescent probe was prepared by mixing 1.2 ml fluorescein in 16.8 ml assay buffer. The excitation and emission wavelength were set at 485 nm and 528 nm. 25 µl 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 [13]. All samples were analyzed in duplicates at three different dilutions. 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.

Antibacterial Activity

Microorganisms

The bacteria used in this study were *Bacillus subtillis* ATCC 6633, *Micrococcus sp.* ATCC 700405, *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 11229, *Shigella sonnei* ATCC 9290 and *Proteus vulgaris* ATCC 6380.

Bacterial strains

The antibacterial activity of fruit peels extracts was evaluated using five Gram-positive bacteria and five Gram-negative bacteria. All of the bacterial strains were obtained from Laboratory of Microbiology, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Malaysia. The microorganism strains were inoculated in universal bottle containing nutrient broth at 37°C for 24 h in water bath (Merck, Germany). The optical density (O.D) of the bacterial suspension was adjusted turbidometrically to 1.1 O.D at wavelength of 600nm.

Antibacterial Disc Diffusion Assay

The screening of the extracts on antibacterial activity was carried out by determining the diameter zone of inhibition using paper disc (6 mm in diameter, Whatman No. 1) diffusion method. The sterile discs impregnated with 25 µl of extract solution with varied concentration (25 to 100 mg/ml extract) were placed in inoculated agar that has been swabbed with adjusted bacteria. The zones of growth inhibition around the discs were measured after 18 to 24 h of incubation at 37°C. Chloramphenicol (10 µg/disc) was used as standard. The controls were prepared using the same solvents but without extracts. The sensitivity of the microorganism species to the crude extracts was determined by measuring the sizes of inhibitory zones (including the diameter of disc) on the agar surface around the disc, and values <6.5 mm were considered as not active against microorganisms.

Statistical Analysis

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

Table 1. Total phenolic and total flavonoid content of aqueous extracts of selected fruit peels

	Total phenolic content (mg GAE/g EW)	Total flavonoid content (mg QE/g EW)
Psidium guajava	381.32 ± 10.61^{b}	39.36 ± 3.56^{c}
Mangifera indica	480.11 ± 20.41^{a}	50.73 ± 0.74^{a}
Malus sylvestris	199.26 ± 8.15^{c}	44.62 ± 1.39^{b}
Citrus sinensis	357.23 ± 23.91^{b}	22.79 ± 2.29^{d}

The values of total phenolic content and total flavonoid content were with the mean ± standard deviation (n=5). Data with different letter in the same column is significantly difference at the level $p < 0.05$.

Results and Discussion

Total Phenolic Content and Total Flavonoid Content

The total phenolic content of the extracts were determined using Folin-Ciocalteu (FC) colorimetric method. This method allows the estimation of all the flavonoids, anthocyanins and other phenolics compounds (non-flavonoids) present in the samples. The TPC of respective fruit peel extracts is shown in Table 1. *M. indica* and *M. sylvestris* peel extracts have the highest and lowest TPC respectively. Meanwhile, there is no significant different between *C. sinensis* and *P. guajava*.

The total flavonoid content was performed by precipitating the crude extract with aluminum chloride ($AlCl_3$). The Al^{3+} will bind with the ketone and hydroxyl group of the flavonoids through electron transfer reaction and produced intense yellow colour when observed under UV Spectrophotometer at the maximum absorbance of 510 nm [14, 15]. Flavonoid is considered to be the largest group of naturally occurring phenols and it is estimated that 2% of all the carbon photosynthesized by plants is converted into this form [16]. Referring to Table 1, the TFC of aqueous extracts were in order of *M. indica* < *M. sylvestris* < *P. guajava* < *C. sinensis*. The high TFC in *M. sylvestris* indicates that, most flavonoid substances present in its tissues are polar and high solubility in water medium.

Recently, plants have been pre-treated with various methods to obtain more bioactive components including heat treatment, far-infrared radiation, ultrasound-assisted, fermentation and alkaline hydrolysis [6, 17]. In this study, a simple heat-drying at 60ºC was conducted in all fruit peel samples in order to remove the moisture content, destroy the degradative enzymes, retard microorganisms' growth other than to cleaved more bioactive components. 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). Phenolic compounds are mostly polar, thus increasing solvent polarity will increased the extraction yields [18, 19]. 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 [6, 20]. According to Oboh (2005) [21], high molecular weight phenolics like tannins and lycopene will breakdown to simple phenols when exposed to high temperature, hence resulting in an increased of free hydroxyl groups.

DPPH Radical Scavenging Assay

DPPH assay is used to determine the scavenging potential of antioxidant extract based on its capability as hydrogen donator and electron transfer. The reaction between antioxidant compounds with the stable DPPH radical will caused reduction in absorbance and decolourisation of DPPH to light yellow [22]. The radical scavenging activity of the reference compounds and the selected fruit peel extracts are presented in Table 2. EC_{50} is defined as the concentration of antioxidant necessary to scavenge 50% of DPPH radicals. According to Maisuthisakul *et al*. (2007) [23], a lower value of EC_{50} corresponds to a higher antioxidant activity of the plant extract. Therefore, the scavenging activity of aqueous extracts were in the order of *Mangifera indica* > *Psidium guajava* > *Citrus sinensis* ≥ *Malus sylvestris* with no significant difference between *C. sinensis* and *M. sylvestris*. The potent scavenging activities of all extracts may be contributed by their phenolic compounds, as we managed to identify strong correlation with the total phenolic content (Table 3). However, the weak correlation with total flavonoid content suggested that other phenolics constituents (non-flavonoids) such as carotenoids, tannins, phenolic acids, may contribute to the main antioxidant

sources. Kuda and Ikemori (2009) [24] claimed that other hydrophilic compounds such as peptides, vitamins and also the occurrence of Maillard reaction may also influenced the antioxidant activities.

Ferric Reducing Antioxidant Power Assay

The reducing antioxidant power of sample extract 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[25]. Table 2 showed the FRAP valued is in order of *Mangifera indica* > *Citrus sinensis* > *Psidium guajava* > *Malus sylvestris* where *M. indica* extracts possessed ferric reducing activity by almost three fold of activity by *M. sylvestris* extract.

Our findings however, showed significantly higher results than previous studies. In accordance to Guo *et al.* (2003) [26], FRAP value for *P. guajava, M. indica* and *C. sinensis* peel extracts were 10.24 mM/100g, 10.13 mM/100g and 5.69 mM/100g respectively using water extraction. Meanwhile, *M. sylvestris* peel extracts was in the ranged of 7.28 to 12 mM/100g by using ethanol as solvent medium [27]. As FRAP measures the activity of hydrophilic antioxidant, the usage of polar solvent would be more useful, and yet, applying heat during extraction would be more significant. Similarly, Alothman *et al*. (2009) [28] reported that aqueous extraction of *P. guajava* possessed higher FRAP than other organic solvent. Furthermore, the FRAP values were well-correlated with the total phenolic content (Table 3) indicating that most extracted phenolic compounds have good redox property.

Table 2. Ferric Reducing Antioxidant Power (FRAP), DPPH radical scavenging activity (EC_{50}), Beta-carotene inhibition and Oxygen Radical Absorbance Capacity (ORAC) values of selected fruit peels

TYPES	EC_{50} (mg/ml)	FRAP (mM/100g)	ORAC (µM TE)	Beta-Carotene (% inhibition)
Citrus sinensis	0.637 ± 0.026^{a}	27.59 ± 1.464^{b}	982.51 ± 40.34^{a}	55.18 ± 3.889^{a}
Malus sylvestris	0.665 ± 0.032^{a}	13.91 ± 2.135^{c}	892.76 ± 55.63^{b}	49.05 ± 2.146^{b}
Mangifera indica	0.144 ± 0.004^{c}	32.98 ± 1.340^{a}	1000.88 ± 71.08^{a}	56.81 ± 7.735^{ab}
Psidium guajava	0.459 ± 0.003^{b}	22.59 ± 1.550^{b}	856.76 ± 42.03^{b}	58.67 ± 3.389^{a}
Ascorbic Acid	0.046 ± 0.001	-	-	-
BHA	0.115 ± 0.002	-	-	73.59 ± 4.245
BHT	-	-	-	71.98 ± 0.924
Trolox	-	44.45 ± 1.571	-	-

Values, mean ± SD (n = 5, determinations in triplicate). Data with different letters in the same column are significantly different at $p < 0.05$

Table 3. The correlation between Total Phenolic and Total Flavonoid Content towards different antioxidant assays (EC_{50}, FRAP, ORAC and ß-carotene bleaching)

	EC_{50}	FRAP	ß-carotene	ORAC
TPC	0.723	0.960	0.737	0.0.53
TFC	0.276	0.249	0.140	0.430

Beta-carotene Bleaching Assay

This anti-lipid peroxidation was carried out in oil-in-water emulsion condition where initially, hydroperoxides were produced at high temperature by linoleic acids, which then react and damaging the Beta-carotene compound. The antioxidant compounds in the extracts can reduce the extent of Beta-carotene destruction by neutralizing the free radical formed in the system[29]. The inhibitory effect of aqueous extracts towards lipids peroxidation was measured by the degradation rate of Beta-carotene[28]. All extracts showed lower antioxidant activity than the corresponding standards (BHA and BHT). *M. sylvestris* peel extract had the lowest percent bleaching inhibition however no significant difference between *M. indica* and *P. guajava* (**Table 2**). The moderate correlations between percent inhibition with TPC showed in **Table 3** indicates that phenolic compounds were not the only antioxidant contributor presence in the extracts, but other water-soluble antioxidant compounds might also influenced the inhibition. Similarly, Othman *et al*. (2007) [28] claimed that water extracts showed better inhibition than other solvent.

Table 4. Antibacterial activities (diameter of inhibition zone, mm) of aqueous crude extracts of selected fruit peels

Bacteria	Chloram-phenicol 10µg	Aqueous extracts samples (Inhibition zone ,mm)											
		Malus sylvestris			*Psidium guajava*			*Mangifera indica*			*Citrus sinensis*		
		[a]25	50	100	25	50	100	25	50	100	25	50	100
Bacillus subtilis (ATCC 6633)	20 ± 0.5	-	-	-	7.0± 0.1	7.5±0.2	8.0± 0.0	-	-	-	-	-	7.5±0.2
Micrococcus species (ATCC 700405)	35 ± 0.5	-	-	-	-	7.0± 0.1	12.5± 0.5	-	-	8.0±0.2	-	-	6.5±0.1
Staphylococcus aureus (ATCC 43300)	16 ± 0.4	-	-	-		-	8.0±0.2	6.5±0.0	7.0±0.2	9.0±0.3	-	-	8.5±0.0
Escherichia coli (ATCC 11229)	-	-	-	-	-	-	-	-	-	-	-	-	-
Shigella sonnei (ATCC 9290)	21 ± 0.4	-	-	-	-	-	-	-	7.0±0.1	7.5±0.0	-	-	-
Proteus vulgaris (ATCC 6380)	10 ± 0.3	-	-	8.0±0.4	-	-	-	-	8.5±0.2	9.0±0.2	-	-	-

[a] The concentration of each extracts were 25, 50 and 100 miligram per mililiter (mg/ml).

Oxygen Radical Absorbance Capacity Assay (ORAC)

This method is used to measure the antioxidant activity possessed by both hydrophilic (phenolics and flavonoids) and lipophilic (carotenoids) antioxidant. ORAC is different than other assay as there are free radicals or oxidants being applied [30]. The oxidative degradation occurred through the spontaneous decomposition of AAPH and produces peroxyl 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. The ORAC values in this study were in the order of *Mangifera indica* > *Citrus sinensis* > *Malus sylvestris* > *Psidium guajava*. However, ORAC assay showed moderate correlations with both TPC and TFC (Table 3).

Antibacterial Activity Assay

The antibacterial properties of crude extracts of *P. guajava*, *M. indica*, *M. sylvestris* and *C. sinensis* peels against six common foodborne bacteria were assessed quantitatively by determining the diameter of inhibition zones as shown in Table 4. *P. guajava*, *M. indica* and *C. sinensis* showed significant inhibition towards all Gram-positive bacteria. *M. indica* also showed effectiveness against Gram-negative bacteria. *M. sylvestris*, however, showed inactivity in all bacteria strains except against *Proteus vulgaris*. *Escherichia coli* was intrinsic resistant pathogen that not only insensitive towards all extracts but also resistance to the standard.

In accordance to Ibezim (2005) [31], Gram-negative bacteria usually resistant to many antibiotics that are effective against Gram-positive bacteria because they are associated with permeable membrane that limiting the transport system and the target sites of the antibiotics. Therefore, a higher concentration of antibacterial agent was essential to inhibit the Gram-negative bacteria but, the high dosage cannot be tolerated by human beings[32]. The good bacterial inhibition displayed by aqueous extract of fruit peels indicates that p active bioactive compounds were polar and thus dissolved readily in the water. In accordance to previous studies, extraction using highly polar medium displayed better antibacterial activities than medium and low polarity medium [33, 34]. Furthermore, boiling might also enhanced the high antibacterial activity as the heat increased the complexes metallic ions in the distilled water with macro-molecules such as saponins and tannins [35].

The effectiveness as antibacterial agent depends on the types of phenolics present especially tannins and flavonoids. Flavonoids that contained hydroxyl group in ring B including quercetin-3-O-rhamnoside, myricetin, morin and any isoflavones were claimed to be potent antibacterial agent [36,37]. Some flavonoids showed weak activity or no activity against bacteria such as apigenin[38]. Yet, phenolic compound like carotenoids, which are responible for the bright colour were abundantly distributed in *C. sinensis* peel, did not pssess any antibacterial activity.

Conclusion

The study showed that fruit peels contained high amount of phenolics and flavonoids and strong antioxidant and antibacterial activities. Present study also showed that *Mangifera indica* peel had the most promising antioxidant agent as potent as the synthetic antioxidants BHA and BHT. The peel of *Mangifera indica* also showed potent antibacterial activities against both Gram-positive and Gram-negative bacteria. Further study on the isolation of individual compounds and the effect of fruits' acidity and colour pigment on fruit peels is necessary.

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