

Research Article

Antioxidant Capacities of Peel, Pulp, and Seed Fractions of *Canarium odontophyllum* Miq. Fruit

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Antioxidant capacities of ethylacetate, butanol, and water fractions of peel, pulp, and seeds of *Canarium odontophyllum* Miq. (CO) were determined using various *in vitro* antioxidant models. Ethylacetate fraction of peel (EAFPE) exhibited the highest total phenolic (TPC), total flavonoid content (TFC), and antioxidant activities compared to pulp, seeds, and other solvent fractions. Antioxidant capacities were assayed by total antioxidant capability, 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical activity, ferric reducing antioxidant power (FRAP), and hemoglobin oxidation assay. Total phenolic content of ethylacetate fractions was positively correlated with the antioxidant activity. This is the first report on the antioxidant activities from CO fruit fractions. Thus, EAFPE can be used potentially as a readily accessible source of natural antioxidants and as a possible pharmaceutical supplement.

1. Introduction

Antioxidants are the compounds that when added to lipids and lipid-containing foods can prolong the shelf-life by retarding the process of lipid peroxidation during processing and storage. Synthetic antioxidants such as butylated hydroxy toluene (BHT) have restricted usage in foods, because it is reported to be carcinogenic [1]. Hence, the importance for utilizing antioxidants from plant origin has received much attention recently. Various extracts from fruits have been recognized to possess beneficial effects against free radicals in biological systems as natural antioxidants [2]. Many studies have shown positive correlation of the increased dietary intake of natural phenolic antioxidants with the reduced coronary heart disease and cancer mortality, as well as with longer life expectancy [3].

Canarium odontophyllum Miq. belongs to the family Burseraceae and is classified as a underutilized fruit due to lack of promotion and as an economic potential which has not been fully explored [4]. The fruit is found in the tropical

rain forest of Sarawak, Malaysia, and is commonly called “dabai” and consumed by the local communities. The fruits are oblong in shape (Figure 1(a)) measuring 3-4 cm and weighing 10–13 g. The fruit peel is purple in color with yellow pulp (Figure 1(b)) and a single three-angled seed (Figure 1(c)). Pulp and seed contribute to bulk of the fruit weight comprising 46% and 44% while peel constitutes 10%. The fruits are highly seasonal (November-January) and hard to be consumed as such. Hence, fresh fruits are usually soaked in warm water for five to ten minutes to soften them for consumption. The fruit is highly nutritious and rich in minerals, proteins, carbohydrates, and fat [5].

For the first time, this study investigated the antioxidant activities of *C. odontophyllum* fruit fractions, and demonstrated the potent bioactivities of the extracts suitable to be used as natural antioxidants. Different fruit parts, namely peel, pulp, and seeds were chosen in an attempt to make systematic comparisons among their antioxidant capacities and to identify the fraction with high antioxidant activity for further studies. In addition, correlations between total

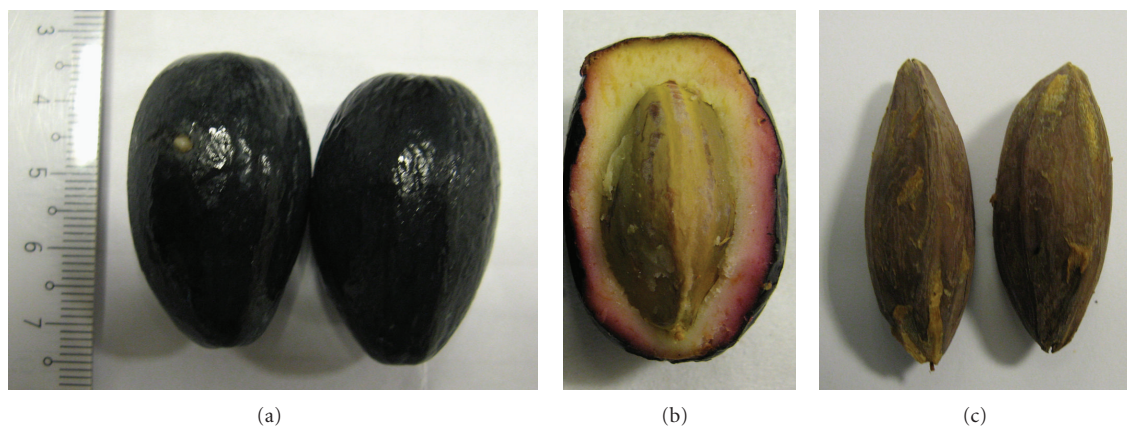


FIGURE 1: Fruits of *Canarium odontophyllum* (a), pulp (b), and seed (c). Unit of measurement is in cm.

phenolics, flavonoid content, and antioxidant capacities were also determined.

2. Materials and Methods

2.1. Plant Material. Fresh fruits of *Canarium odontophyllum* Miq. (20 kg) at the mature stage were provided by Agriculture Research Centre, Department of Agriculture, Sarawak, Malaysia. The fruits were transported in ice box by airmail on the same day to Universiti Putra Malaysia. Fruits were chosen for uniformity in shape and color, washed carefully with tap water, and air dried. The fruit peel, pulp, and seeds were manually separated, then dried in oven at 60°C for 24 h, and finally they were grounded into powder using a blender. The moisture content of the fresh peel, pulp, and seeds was determined to be 65.5 ± 2.1 , 53.5 ± 4.2 , and $23.8 \pm 1\%$, respectively.

2.2. Chemicals and Reagents. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine (TPTZ), aluminium chloride, quercetin, gallic acid, BHT, trichloroacetic acid, thiobarbituric acid, sodium azide, Tris-HCl buffer, phosphate buffer, and Heps were obtained from Sigma-Aldrich Co. (MO, USA). Folin-Ciocalteu reagent and hydrogen peroxide were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade.

2.3. Extraction. Dried powder (10 g) of CO peel, pulp, and seeds was extracted separately using a rotary shaker (Unimax 1010, Heidolph, Germany) at 4000 rpm with 100 mL of 50% ethanol at 30°C for 5 h. The extract was then filtered, and concentrated using a rotary evaporator (Buchi, Flawil, Switzerland). The concentrated extract was then partitioned sequentially with 100 mL of ethyl acetate, butanol, and water. The fractions obtained from peel, pulp, and seeds were collected separately, concentrated, freeze dried, and stored at -20°C until further use. All the freeze-dried extracts were dissolved in 50% ethanol to get the appropriate

concentrations. The extraction yields of peel, pulp, and seed were 24.6, 18.4, and 15.1%, respectively.

2.4. Determination of Total Phenolic Content. Total phenolic content of each fraction obtained by the above method was determined according to the method of Singleton and Rossi [6] and then expressed as milligram/gram gallic acid equivalents (GAE). In brief, 100 μL -aliquot of the samples (1 mg/mL) was added to 2 mL of 20 g/L Na_2CO_3 solution. After 2 minutes of incubation, 100 μL of 50% Folin-Ciocalteu reagent was added and the mixture was then allowed to stand for 30 min at 25°C. The absorbance was measured at 750 nm using a spectrophotometer (UV 1601, Shimadzu Co., Ltd., Kyoto, Japan). The blank consisted of all reagents and solvents without the sample. The total phenolic content was determined using the standard gallic acid calibration curve.

2.5. Determination of Total Flavonoid Content. Total flavonoid content of the fractions was measured following the aluminum chloride colorimetric assay described by Liu et al. [7]. An aliquot (2 mL) of the sample was mixed with 0.2 mL of 5% sodium nitrite. After 5 minutes, 0.2 mL of 10% aluminum chloride was added to the mixture. Following 6 minutes, 2 mL of 1 M sodium hydroxide were added to the mixture. The final volume of the reaction mixture was made up to 5 mL with 50% ethanol. Absorbance was measured at 510 nm against a blank. The total flavonoid content was determined using a standard curve of quercetin (0–50 mg/mL) and the results were expressed as quercetin equivalents.

2.6. Analyses of Antioxidant Activities

2.6.1. DPPH Radical Scavenging Activity. DPPH radical scavenging activities of the fractions were determined following the method of Blois [8] with some modifications. Different concentrations (5, 10, 20, and 40 $\mu\text{g}/\text{mL}$) of the fractions and BHT were placed in different test tubes and were mixed with 1 mL of 0.2 mM DPPH (dissolved in methanol). The reaction mixture was shaken vigorously and incubated at 28°C in

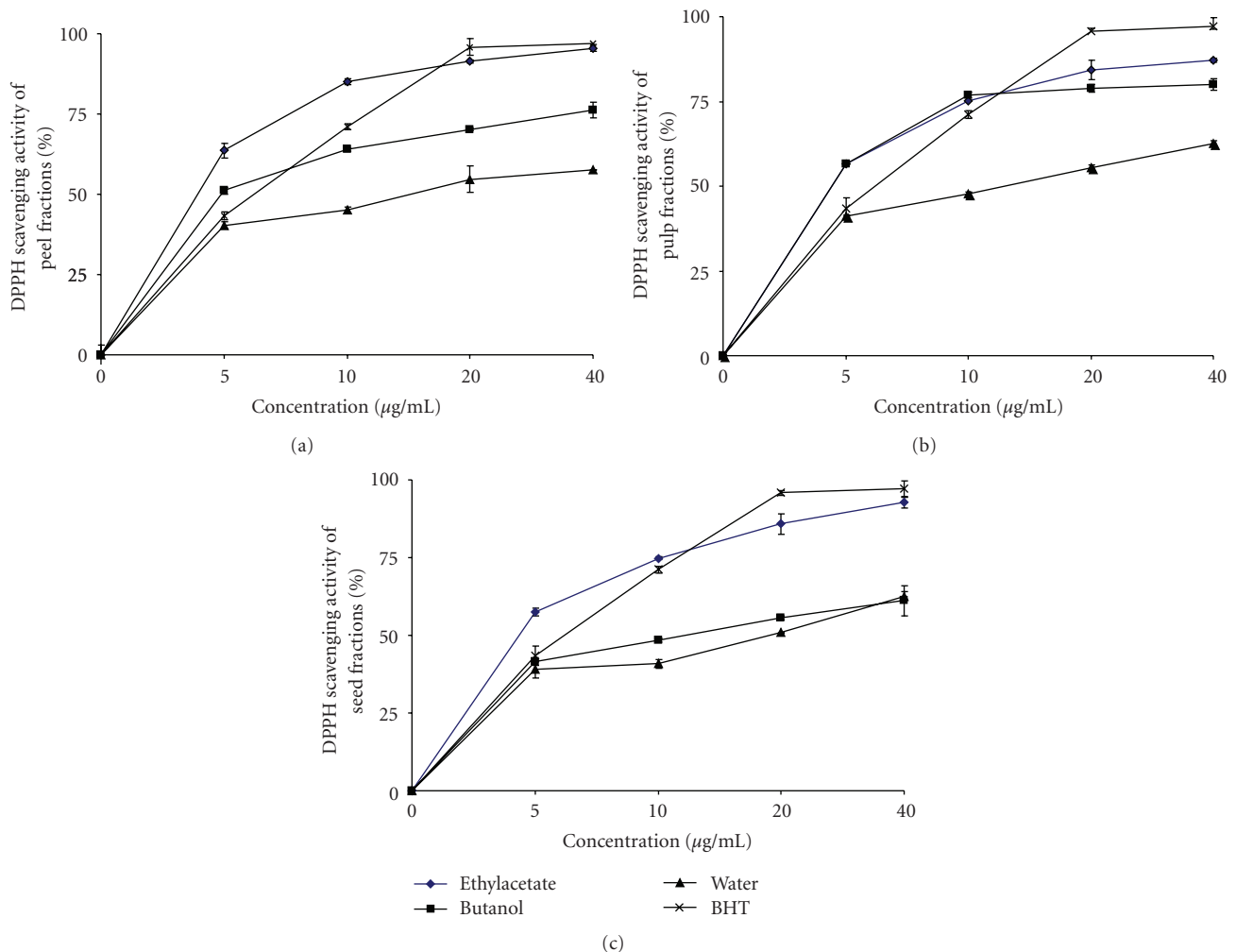


FIGURE 2: DPPH radical scavenging activity of *Canarium odontophyllum* peel fractions (a), pulp fractions (b), and seed fractions (c).

a dark room for 40 min. The control was prepared as above without any extract, and methanol was used for the baseline correction. The changes in absorbance were measured at 517 nm using a spectrophotometer. The inhibition of DPPH radicals was calculated as scavenging activity (%) = $(\text{Control OD} - \text{sample OD} / \text{control OD}) \times 100$.

2.6.2. Total Antioxidant Capacity by Phosphomolybdenum Method. Total antioxidant capacities of the CO fractions and BHT were determined by the method of Prieto et al. [9]. An aliquot (0.1 mL) of the sample fractions at different concentrations (5, 10, 20, and 40 µg/mL) was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixture was covered and incubated at 95°C for 90 min. After the mixture was cooled, the absorbance was measured at 695 nm against blank. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for dissolving the sample, and it was incubated under the same conditions. The total antioxidant activity was expressed as the absorbance value at 695 nm.

A higher absorbance value indicates a higher antioxidant activity.

2.6.3. Ferric Reducing Antioxidant Power (FRAP) Assay. Ferric reducing antioxidant power assay of sample extract was performed according to method of Benzie and Strain [10] with slight modifications. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6) : 10 mM TPTZ solution in 40 mM HCL : 20 mM ferric chloride solution, in proportion of 10 : 1 : 1 (v/v/v). An aliquot (50 µL) of appropriately diluted sample extract was mixed with 3 mL of freshly prepared FRAP reagent and mixed thoroughly. The reaction mixture was then incubated at 37°C for 30 minutes. Absorbance of the reaction mixture was read at 593 nm against a blank. A higher absorbance value indicates higher antioxidant activity.

2.6.4. Hemoglobin Oxidation Assay. Fasting venous blood (10 mL) from healthy volunteers (aged 20–30 years) were collected in EDTA tubes (0.4 g/L). As described by Chu and Liu [11], the plasma was centrifuged at 1600 xg for 20 min

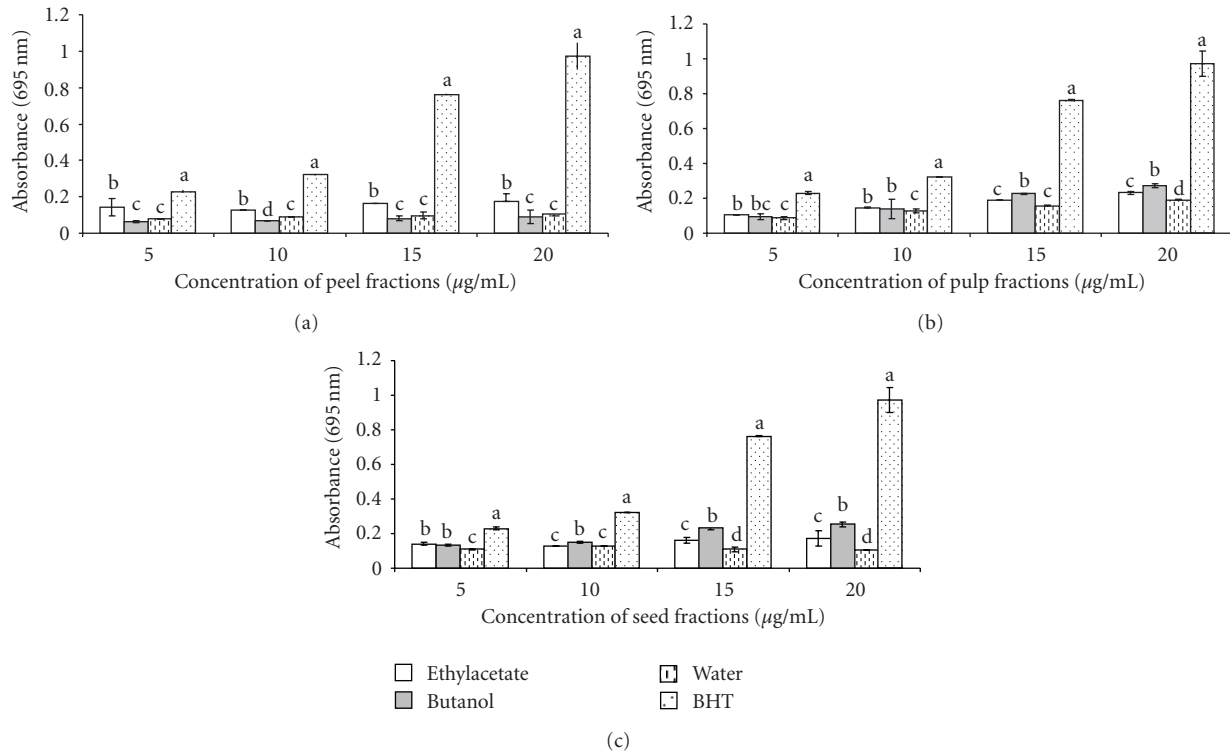


FIGURE 3: Total antioxidant capacity of *Canarium odontophyllum* peel fractions (a), pulp fractions (b), and seed fractions (c). Higher absorbance value indicates higher antioxidant activity. Different letters above the bars for the same concentration indicate statistically significant differences at $P < .05$.

at 4°C for separation of red blood cells (RBC). Red blood cells were washed three times with phosphate buffer saline (PBS).

Hemoglobin oxidation was performed as previously described by Rodríguez et al. [12] with slight modifications. The experiment was carried out within a day of blood withdrawal. The red blood cells (RBCs) were gently resuspended with PBS to obtain 5% of RBC and preincubated at 37°C for 10 min in the presence of 1 mM sodium azide. Subsequently, 1.6 mL of RBC was transferred to test tube for experimental analysis. All test tubes except control were added with 10 mM of H₂O₂ and with or without the addition of sample extracts (5, 10, and 15 µg/mL, 0.2 mL), while control test tube received only 0.2 mL of 50% ethanol. Following 60 min incubation at 37°C, the mixture was kept for 60 sec in an ice bath and then centrifuged at 1853 x g for 10 min at 4°C. Malondialdehyde (MDA) levels were measured using TBA assay as described by Buege and Aust [13]. The percentage inhibition of the fractions against hemoglobin oxidation was also calculated using the following equation: (%) = (OD of H₂O₂ induced haemoglobin—sample OD/OD of H₂O₂ induced haemoglobin) × 100.

2.7. Statistical Analysis. Data were expressed as means ± standard deviations (SDs) of three replicate determinations and then analyzed by SPSS V.13 (SPSS Inc., Chicago, USA). One way analysis of variance (ANOVA) and Duncan's New Multiple-range test were used to determine the differences

among the means. P -values <.05 were considered to be significantly different.

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Contents. Table 1 shows the total phenolic and flavonoid contents of each fraction of CO fruit. Great variation in phenolic content was observed in peel, pulp, and seed fractions of CO ranging from 68 ± 1.2 – 10 ± 1.5 mg GAE/g in ethyl acetate fractions, 35 ± 1.6 – 4 ± 0.4 mg GAE/g in butanol fractions, 18 ± 4.8 – 3 ± 0.2 mg GAE/g in water fractions. Ethyl acetate fraction of peel (EAFPE) had the highest (68 ± 1.2 mg GAE/g) phenolic content, while the lowest (3 ± 0.2 mg GAE/g) was observed in water fraction of seed. Phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activity [14]. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu (FC) reagent [6]. However, it should be also noted that some chemical groups of proteins, organic acids, and sugars present in the extracts can also react with FC reagent and therefore can interfere with the results [15].

Ethyl acetate was used to extract medium polar flavonoids and glycosides while butanol and water were used for extracting polar compounds like phenolic acids, aglycones, glucosides, and sugars [16, 17]. In our previous work, higher phenolic content was obtained in ethyl acetate

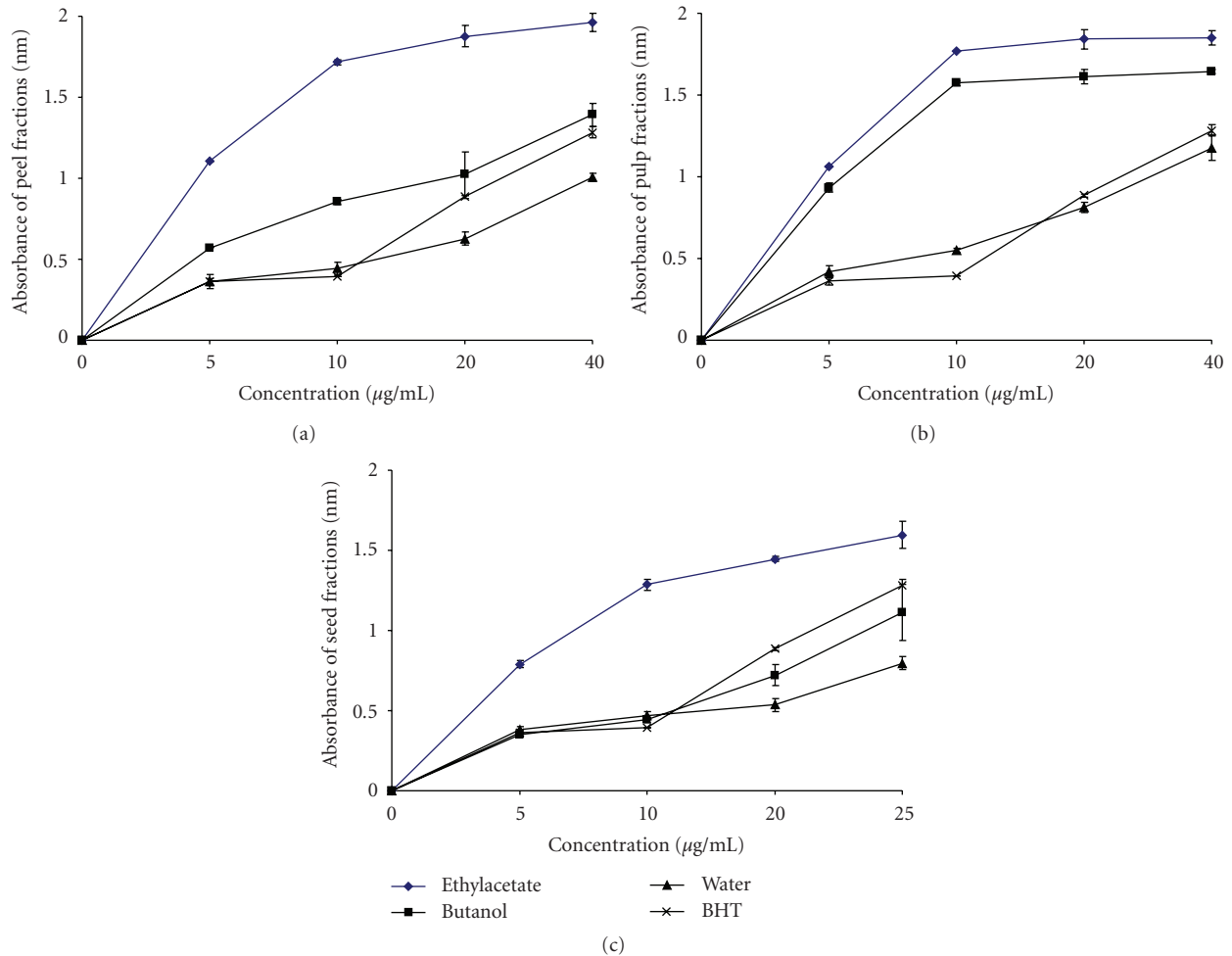


FIGURE 4: Reducing power of *Canarium odontophyllum* peel fractions (a), pulp fractions (b), and seed fractions (c). Higher absorbance value indicates higher antioxidant activity.

TABLE 1: Total phenolic and flavonoid content of *Canarium odontophyllum* fractions.

Sample	Solvent	TPC*	TFC*
Peel	Ethyl acetate	68 ± 1.2 ^a	173 ± 9.9 ^a
	n-Butanol	35 ± 1.6 ^b	28 ± 0.5 ^d
	Water	18 ± 4.8 ^c	20 ± 0.4 ^e
Pulp	Ethyl acetate	14 ± 0.5 ^d	134 ± 1.5 ^b
	n-Butanol	14 ± 0.2 ^d	29 ± 2.7 ^d
	Water	5 ± 0.4 ^f	18 ± 0.5 ^f
Seed	Ethyl acetate	10 ± 1.5 ^e	115 ± 3.8 ^c
	n-Butanol	4 ± 0.4 ^f	22 ± 1.6 ^e
	Water	3 ± 0.2 ^g	18 ± 0.1 ^f

* Values are mean ± standard deviation of three replicate analyses. TPC: total phenolics content expressed as mg gallic acid equivalent/g; TFC: total flavonoids content expressed as mg quercetin equivalent/g. For each treatment (TPC and TFC), the means in a column followed by different letters were significantly different at $P < .05$.

fraction compared to butanol and water [18, 19]. Thus, it is necessary to extract phenolic compounds effectively from CO prior to the further evaluation of antioxidant activity.

Flavonoids are one of the most diverse and widespread group of natural compounds and are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [3, 14]. The total flavonoid content was found to be the highest in EAFPE (173 ± 9.9 mg/g QE) and the lowest (18 ± 0.1 mg/g QE) in water fraction of seeds.

3.2. DPPH Radical Scavenging Activity. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity. In DPPH radical scavenging assay, antioxidants react with DPPH (deep violet color), and convert it to yellow coloured α, α -diphenyl- β -picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the antioxidant [8, 15]. The DPPH radical scavenging activity of all the fractions from CO increased as concentration increased (Figures 2(a)–2(c)). Ethyl acetate fraction of peel exhibited the highest scavenging activity (95.5 ± 1%) compared to other fractions at a concentration of 40 µg/mL, and was equal to scavenging

activity of BHT (Figure 2(a)). Ethyl acetate fractions of pulp (Figure 2(b)) and seed (Figure 2(c)) also exhibited high scavenging activity of $87 \pm 0.7\%$ and $85.8 \pm 3.2\%$ at $40 \mu\text{g/mL}$ concentration. All the butanol fractions exhibited moderate scavenging activity of $79.9 \pm 1.7\%$ in the pulp being the highest, while the lowest ($17.3 \pm 0.3\%$) was observed in the seed. Low scavenging activity was noticed in all the water fractions. It has been found that phenolics, flavonoids, and tocopherols scavenge DPPH radicals by their hydrogen donating ability [15, 18]. The results obtained in this investigation reveals that all the fractions of CO could act as free radical scavengers, which might be attributed to their electron donating ability.

3.3. Total Antioxidant Capacity. The total antioxidant capacities of CO fractions was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm [9]. A high absorbance value of the sample indicates its strong antioxidant activity. Figures 3(a)–3(c) show the total antioxidant capacities of CO fractions and BHT. All the fractions showed a good total antioxidant capacity, which was concentration dependent. The total antioxidant activity of EAFPE at $20 \mu\text{g/mL}$ was 0.17 ± 0.03 (Figure 3(a)), significantly higher ($P < .05$) than butanol and water fractions. Interestingly, butanol fraction of pulp (Figure 3(b)) and seed (Figure 3(c)) showed higher value than other fractions. However, the total antioxidant activity of BHT at all concentrations tested was highest than CO fractions. Previously, Jayaprakasha et al. [17] indicated that the total antioxidant activity of citrus was due to the presence of phenolics and flavonoids. The total antioxidant capacity in the present investigation may be attributed to total phenolic and flavonoid contents.

3.4. Ferric Reducing Antioxidant Power (FRAP). Ferric reducing antioxidant power is widely used in evaluating antioxidant activity of plant polyphenols. Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox-linked colorimetric reaction [15]. This assay is relatively simple and easy to conduct. FRAP assay measures the reducing potential of antioxidant to react on ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex and produce blue color of ferrous form which can be detected at absorbance 593 nm [10]. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction [20]. In the present study, CO peel exhibited the highest reducing power followed by seed and pulp. Among CO peel, the ethyl acetate fraction exhibited a strong reducing power and was higher than BHT, as shown in Figure 4(a). At $40 \mu\text{g/mL}$, the reducing power ability of EAFPE and BHT was 1 ± 96.05 and 1.2 ± 0.03 . All the fractions of pulp and seeds showed higher FRAP values compared with BHT. The reducing power of pulp fractions (Figure 4(b)) was lower than seed fractions (Figure 4(c)). Similar observations have been reported by other authors, where in the FRAP values of peel was higher, followed by

seed and pulp fractions [2]. The reducing power of CO fruit fractions are probably due to the action of hydroxyl group of the phenolic compounds which might act as electron donors.

3.5. Hemoglobin Oxidation Assay. In this study, hemoglobin oxidation assays was chosen as they mimic human biological system [21]. Many *in vitro* models have been tested to determine the effectiveness of antioxidants against free radicals; among them, hemoglobin in the erythrocytes has become a very useful assay to evaluate the effects of free radicals and antioxidants on cellular system [12]. Oxidative damage to hemoglobin by exposure to hydrogen peroxide is a primary mechanism to induce specific structural changes and might contribute to hemoglobin-mediated toxicity in diseases linked to oxidative stress [22]. The result showed that all the fractions had a good protective effect against hydrogen peroxide induced hemoglobin oxidation (Table 2). The percentage inhibition of hemoglobin oxidation varied significantly in all the tested fractions with the highest activity ($50 \pm 4.3\%$) observed in ethyl acetate seed fraction, while the lowest (33 ± 1.6) was in water pulp fraction.

Malondialdehyde (MDA) is the by-product of peroxidation of phospholipids and generally regarded as a marker for oxidative stresses, rendering its determination in biological samples particularly interesting [12]. The MDA produced from hemoglobin oxidation treated with ethyl acetate fraction from seed was lower than pulp and peel fractions at a concentration of $5 \mu\text{g/mL}$. The MDA levels of H_2O_2 -induced hemoglobin oxidation treated with different concentrations (5, 10, and $15 \mu\text{g/mL}$) of the fraction decreased compared to H_2O_2 -induced hemoglobin. However, many of the fractions had protective effect against H_2O_2 -induced oxidation only at lower concentration. A similar finding reported by Li [23] showed that lotus germ oil had pro-oxidation effect at higher concentrations.

Ethyl acetate fractions of the peel contain higher amount of phenolic compounds and it is not surprising that peel extract displays higher antioxidant activity than pulp and seed. Besides, synergistic action among different antioxidants in the fraction can also be considered. As given in Table 1, the total phenolic content of EAFPE was nearly five and seven times higher than pulp and seeds. Additionally, the flavonoid contents were also higher compared to pulp and seeds. All these results clearly indicated that peel fraction contains more antioxidants than pulp and seed. Our data was in agreement with Li et al. [24] and Guo et al. [2] who found that fruit peel of mango, kiwifruit, guava, and orange among others contains high concentration of phenolics, flavonols, and antioxidant activities than pulp and seed extracts. Additionally, the total phenolic content of ethyl acetate fraction in the present study was much higher than total phenolic contents of common fruits like apple, grapes, and mandarine (20.5, 15.1, and 15.9 mg/g DW , resp.) [25].

Several studies exhibited a close relationship between antioxidant activities and total phenolic content [7, 17, 20]. A positive correlation ($R^2 = 0.775$) was found between total

TABLE 2: Hemoglobin oxidation inhibitory activity of *Canarium odontophyllum* fractions.

Samples	Fractions	Concentration ($\mu\text{g/mL}$)	MDA (μM)	Inhibition (%)		
Non-induced haemoglobin (control)		—	0.313	—		
H ₂ O ₂ -induced haemoglobin		—	1.42	—		
		Ethyl acetate	5	0.822	42 \pm 7	
			10	0.849	40 \pm 5.9	
			15	0.924	35 \pm 0.2	
			5	0.750	47 \pm 2.2	
		Peel	Butanol	10	0.717	49.4
				15	0.844	47 \pm 0.1
				5	0.752	46 \pm 3.4
			Water	10	0.745	47 \pm 1.7
				15	0.916	48.5
				5	0.725	48 \pm 1.1
		Pulp	Ethyl acetate	10	0.776	45.2
				15	0.813	42 \pm 1.1
				5	0.731	48 \pm 0.4
			Butanol	10	0.742	47 \pm 6.2
15	0.979			44 \pm 1.1		
5	0.937			33 \pm 1.6		
Water	10		0.830	41 \pm 0.7		
	15		0.802	43 \pm 4.8		
	5		0.698	50 \pm 4.3		
Seed	Ethyl acetate		10	0.778	45.1	
			15	0.793	44 \pm 0.5	
			5	0.748	49 \pm 0.3	
	Butanol	10	0.753	46 \pm 4.5		
		15	0.773	45 \pm 2.9		
		5	0.792	44 \pm 4.1		
	Water	10	0.816	42.4		
		15	0.808	43 \pm 0.6		
		5	0.851	40 \pm 0.3		
	BHT	10	1.032	27.2		
15		0.901	36.4			

phenolic contents and DPPH radical scavenging activity. Similar observations ($R^2 = 0.804$) was also observed for FRAP and total phenolic content. Fang et al. [26] reported a similar correlation of $R^2 = 0.741$ between total phenolic content and FRAP. However, a low correlation between DPPH ($R^2 = 0.249$), FRAP ($R^2 = 0.293$), hemoglobin oxidation ($R^2 = 0.313$), and total flavonoids was determined. Liu et al. [7] reported a negative correlation between flavonoid content and antioxidant activity. This clearly indicated that total phenolics are major contributors for antioxidant activity since, they have a high correlation, while flavonoids are not the major contributors for antioxidant activities, since they have a lower correlation in the current study. Further investigations into the identification of phenolic compounds present in the ethyl acetate fraction

of CO are needed to better elucidate their antioxidant activities.

4. Conclusion

In the present study, application of different solvents to extract antioxidant compounds from *Canarium odontophyllum* peel, pulp, and seeds were investigated. This study indicated that the ethyl acetate fraction of peel possessed the highest phenolic and flavonoid contents than other fractions. Also, it exhibited strong antioxidant capacities in all the assays, which were comparable to the commercial BHT antioxidant. This suggests that the peel extract of *C. odontophyllum* can be potentially used as a source of natural antioxidant agent.

Acknowledgments

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