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Antioxidant properties of methanolic extract of *Canarium odontophyllum* fruit

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Abstract: The antioxidant properties of skin, flesh and kernel of *Canarium odontophyllum* fruit were determined. The methanolic extracts of the fruit were screened for their total phenolic content and antioxidant properties. The averaged antioxidant properties (mM TE/g FM) in skin, flesh, and kernel of *Canarium odontophyllum* were 16.46 ± 0.24, 20.54 ± 0.35, and 8.89 ± 0.29, respectively by DPPH assay; 151.24 ± 9.75, 70.58 ± 2.98, and 5.65 ± 0.02, respectively by FRAP assay; and 47.9 ± 0.00, 11.61 ± 1.14, and 3.00 ± 0.00, respectively by β-Carotene bleaching method. The averaged OH scavenging activity (mg DMSOE/mg FM) in skin, flesh, and kernel of *Canarium odontophyllum* were 43.33 ± 13.85, 7.81 ± 1.42, and 3.31 ± 0.80, respectively. While averaged total phenolic content (mg GAE/100g FM) were 387.5 ± 33.23, 267.0 ± 4.24, and 51.0 ± 0.00 for skin, flesh, and kernel respectively. Antioxidant activities were positively correlated with the total phenolic content ($0.71 \leq r \leq 0.84$).

Keywords: Phenolic, DPPH, β-carotene, FRAP, hydroxyl scavenging activity

Introduction

Reactive oxygen species (ROS) are free radicals capable of causing damage to DNA, have also been associated with carcinogenesis, coronary heart disease, and many other health problems related to advancing age (Cadenas and Davies, 2000; Marnett, 2000; Uchida, 2000). The oxidation process by ROS can be minimized through reaction with antioxidants. Antioxidants terminate direct ROS attacks and radical-mediated oxidative reactions, and appear to be primarily important in the prevention of many diseases and health problems (Tepe and Sokmen, 2007). The antioxidants are not only needed by our body to combat ROS but are also important as food additives either in synthetic or naturally occurring forms (Lim and Murtijaya, 2007). The synthetic antioxidants possess carcinogenic activity, which necessitates its replacement with naturally occurring ones (Madsen and Bertelsen, 1995). Natural antioxidants tend to be safer and possess anti-viral, anti-inflammatory, anti-cancer, anti-mutagenic, anti-tumor–and hepatoprotective properties (Lim and Murtijaya, 2007).

Antioxidants have been detected in a large number of foods and agricultural products, including cereal grains, vegetables, fruits, and plant extracts (Burits and Bucar, 2000; Kalt *et al.,* 1999; Yu *et al.,* 2002a, b). The rich biodiversity of the tropical rain forest in Sarawak, Malaysia offers an excellent source of fruits and vegetables, especially for the rural communities. *Canarium odontophyllum* or commonly known as Dabai is popular among people living in Sarawak. The domesticated plant can be classified as indigenous fruit and has been consumed by the rural as snack food (Latiff *et al.,* 2000). *Canarium odontophyllum* has high percentage of fat at 26.2% of edible portion compared to other indigenous fruits. The fruit also is a good source of calcium (200 mg), magnesium (106 mg), and phosphorus (65 mg) (Hoe and Siong, 1999). The fruit consist of skin (5-6%), flesh (54-60%) and kernel (35-40%), which are all edible except kernel. Our literature survey did not reveal any report concerning the antioxidant properties of the fruit.

The aim of the present work was to study the antioxidant properties of methanolic extracts of

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C. odontophyllum fruit parts in addition to their total phenolic content. The data gathered is hoped to provide some baseline information which in the end may highlight the potential of the fruit as new source of natural antioxidant-rich food materials with functional properties.

Materials and Methods

Materials

Dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Methanol, Chloroform and Tween 40 were purchased from Fisher Scientific (Langhborough, Leicestershine, UK). Gallic acid was from Sigma-Aldrich (Madrid, Spain) while linoleic acid (60%) was obtained from Sigma Chemical Co. (St. Loius, MO, USA). Ethylenediaminetetraacetic acid (EDTA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and L-ascorbic acid were purchased from Sigma-Aldrich (Deutschland, Germany) while Folin-Ciocalteau reagent and tripyridyl triazine (TPTZ) were obtained from Fluka Biochemica (Buchs, Switzerland). β-carotene was from Sigma-Aldrich Co. (St. Louis, MO, USA). Phosphate buffer, acetate buffer, sodium carbonate and potassium phosphate buffer were purchased from R and M Chemical (Essex, UK). Trolox was from Acros Organics (Geel, Belgium). Butylated hydroxytoulene (BHT) and ferrous ammonium sulphate were obtained from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Potassium chloride (KCl) and $FeCl_3$.$6H_20$ were purchased from AnalaR (Poole, England) while reverse osmosis water was purchased from Cascada (Bucks, UK).

Fruit collection

Canarium odontophyllum fruits were obtained from Sarawak Agriculture Research Centre (ARC), Semongok, Sarawak, Malaysia and were transported to UPM via flight at refrigerated temperature (4°C) in a cold box container. On arrival, fruits with good physical properties were selected and stored at -20°C before further analysis. Prior to analysis the fruits were taken out of freezer, thawed to room temperature and the skin, flesh, and kernel separated using a knife.

Preparation of extract

Methanol extracts of fruit prepared using the method of Kriengsak *et al.* (2006) as modified from Swain and Hillis (1959), were used for total phenolic content and antioxidant activity assays. Four grams of sample from each fraction of *Canarium odontophyllum* fruit (skin, flesh, and kernel) were mixed with 100 mL methanol and homogenized using vortex (EVM-V1000, Erla, Jayu, Korea). The homogenates were kept at 50°C for 2 hours in shaking incubator with revolving speed of 200 rpm (Heidolph, Made in Germany). The homogenates were filtered using Whatman No. 1 filter paper. After filtration, the homogenates were recovered and stored at -20°C prior to analysis. In general, methanol extract are used for determining hydrophilic and lipophilic antioxidant activities from plant materials (Arnao and Acosta, 2001).

β-carotene bleaching method

β-carotene bleaching of extract was determined using the method of Tepe and Sokmen (2007) with a slight modification of the method as per Barriere *et al.* (2001). A stock solution of β-carotene was prepared by dissolving 0.5 mg β-carotene in 1 mL chloroform. Then 25 µL of linoleic acid and 200 mg Tween 40 were added. The chloroform was subsequently evaporated using a vacuum evaporator (Büchi, Flawil, Switzerland). About 100 mL of distilled water saturated with oxygen was added into the mixture with vigorous shaking. Aliquots (2500 µL) of this reaction mixture were transferred into test tubes, and 350 µL portions of the fruit extracts were added before incubating for 48 h at room temperature (23°C). The same procedure was repeated with BHT and blank (350 µL of methanol). After the incubation period, the absorbance of the mixtures was measured at 490 nm with a UV-Vis spectrophotometer (SECOMAM, Domant Cedex, France). The results were expressed in trolox equivalent (TE: mM/g dry mass) using a Trolox standard curve.

FRAP assay

FRAP assay was done according to the method of Kriengsak *et al.* (2006) with some modifications according to Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37°C before being used. Fruit extracts (150 µL) were allowed to react with 2850 µL of the FRAP solution for 30 min in a dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were taken at 593 nm with a UV-Vis spectrophotometer (SECOMAM, Domant Cedex, France). The results were expressed in trolox equivalent (TE: mM/g dry mass) using a trolox standard curve.

DPPH radical scavenging activity

The DPPH assay was carried out according to the method of Kriengsak *et al.* (2006) that was modified from Brand-Williams *et al.* (1995). The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored at -20°C until needed. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm using the UV-Vis spectrophotometer (SECOMAM, Domant Cedex, France). Fruit extracts (150 µL) were allowed to react with 2850 µL of the DPPH solution for 24 h in the dark. Then the absorbance was taken at 515 nm. The results were expressed in trolox equivalent (TE: mM/g dry mass) using a trolox standard curve.

OH scavenging activity assay

OH scavenging activity assay was carried out according to the method of Isao & Lawrence (1990). This assay was performed in a flat cell using a Varian E-9 spectrometer (JEOL-JES-FA100, Tokyo, Japan). Reactions were carried out at 25°C in 20 mM potassium phosphate buffer (pH 7.4) containing 150 mM KC1 (control system). Reactions were started with the flow apparatus by mixing an aerobic solution containing 150 mM KCl, 40 mM phosphate buffer, 40 mM DMPO (standard experiment), 2 mM EDTA, and hydrogen peroxide with an anaerobic solution containing 250 mM KC1 and ferrous ions. The anaerobiosis was attained by gassing with nitrogen gas. The stock solution of ferrous ion (2 mM) was prepared before each experiment by dissolving ferrous ammonium sulfate in anaerobic water. The premixing of ferrous ion with EDTA was avoided because ferrous ions became more auto-oxidizable in the presence of a chelator. Results were expressed in DMSO equivalent (DMSOE: mg/ fresh mass) using a DMSO standard curve.

Determination of total phenolics content

Total phenolics content was determined by the Folin-Ciocalteau method, which was adapted from Azizah *et al.* (2007). About 200 µL of extract and 1500 µL of Folin-Ciocalteu's reagent were combined in test tubes and then mixed well using a vortex (EVM-V1000, Erla, Jayu, Korea). The mixture was allowed to stand for 5 min. Then 1500 µL of 1 N Na_2CO_3 solution was added and mixed well. The solution was incubated at room temperature (23°C) in the dark for 90 min. The same procedure was repeated on trolox (standard) and methanol (control). The absorbance was measured at 725 nm using UV-Vis spectrophotometer (SECOMAM, Domant Cedex, France) and the results were expressed in gallic acid equivalents (GAE: mg/100 g fresh mass) using a gallic acid standard curve.

Statistical analysis

All analyses were run in triplicate. Statistical analysis was conducted using Statistical Package for Social Sciences (SPSS) version 15.0. Data obtained were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was used to test any difference in antioxidant activities resulting from these methods. Duncan's new multiple range test was used to determine significant differences. Differences at $p < 0.05$ were considered to be significant. Correlations among data obtained were calculated using Pearson's correlation coefficient (r).

Results and Discussion

β-carotene bleaching activity

The averaged values for skin, flesh, and kernel were 47.90 ± 0.00 mM TE/g, 11.61 ± 1.14 mM TE/g, and 3.00 ± 0.00 mM TE/g, respectively. Meanwhile, the averaged value for BHT was 19.92 ± 0.59 mM TE/g. ANOVA test showed no significant differences exist between these four averaged values at $p < 0.05$. The antioxidant activity was highest in the skin followed by BHT, flesh, and kernel.

The mechanism of β-carotene bleaching method involved activity of linoleic acid free radical on unsaturated β-carotene until the β-carotene became oxidized and split into a few parts that resulted in the loss of chromophore (orange color) that could be detected by spectrophotometer. However, this mechanism can be inhibited in the presence of antioxidants (Abdille *et al.,* 2005) which inhibit the bleaching of β-carotene via neutralization of linoleic acid free radical and other free radicals (Jayaprakasha *et al.,* 2001).

Reducing power

Reducing power was measured in methanol extracts using FRAP assay showed that the skin had the highest antioxidant activity followed by flesh, BHT, and kernel. The averaged value for skin was 151.24 ± 9.75 mM TE/g, flesh was 70.58 ± 2.98 mM TE/g, kernel was 5.65 ± 0.02 mM TE/g, and BHT was 52.63 ± 2.44 mM TE/g (control). There were significant differences between these values at $p < 0.05$ when ANOVA test was performed.

The principle of FRAP method was based on

the decolorization of blue colored complex ferric-tripyridyltriazine Fe(III) to a dark blue colored ferrous Fe(II) in the presence of antioxidant. The reducing power is expressed when an antioxidant acts as electron donor that reduces the intermediate-oxidized substances produced from lipid peroxidation. Therefore, the component with this reducing power acts as primary and secondary antioxidant (Yen and Chen, 1995).

Results showed that FRAP was highest in the skin followed by flesh, BHT, and kernel of *C. odontophyllum*, which indicates a great potential for the fruit, especially skin as a good natural antioxidant that can replace synthetic antioxidants such as BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole).

Scavenging activity

The scavenging activity of skin, flesh, and kernel of *C. odontophyllum* were 16.46 ± 0.24 mM TE/g, 20.54 ± 0.35 mM TE/g, and 8.89 ± 0.29 mM TE/g, respectively based on DPPH radical assay. There were significant differences between these values at $p < 0.05$. The antioxidant activity in flesh of *Canarium odontophyllum* was highest compared to skin and kernel. The higher scavenging activity in the flesh might be due to the presence of residual skin pigment (due to incomplete removal) that influenced the absorbance value.

DPPH is a free radical which is stable and consists of nitrogen centered in its chemical structure. The reducing purple color 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) to pale yellow *hydrazine* occurs due to reduction process by antioxidant whether in term of hydrogen or electron donation (Pokorny *et al.,* 2001). The substances that are able to act as donor to DPPH free radical was identified as an antioxidant and free radical scavenger (Brand-Williams *et al.,* 1995). DPPH free radical scavenging activity has been reported to show high correlation with inhibition capacity towards lipid peroxidation process (Rekka and Kourounakis, 1991).

According to Haiying *et al.* (2008), Chinese white olive (*Canarium album Raeusch*), a special fruit in Southern China has been used for treating respiratory tract inflammation and food poisoning. Similarly, Wei *et al.* (1999) also showed that the Chinese white olive had the highest phenolics content and strongest antioxidant capacity among 68 Chinese medicinal foods. The fruit also had stronger DPPH radical-scavenging ability than ascorbic acid, exhibiting large value of extrapolation. Therefore, the *C. odontophyllum*, may also possess similar health promoting properties as the *C. album Raeusch* in Southern China.

The determination of DPPH free radical scavenging activity using spectrophotometer can be influenced by other compounds such as carotenoid or other substances that have the same color with DPPH free radical. These substances or compounds can interrupt the reading of absorbance taken for DPPH free radical at 515 nm. Factor such as cloudiness of sample also can influence the absorbance value (Lachman *et al.,* 2005). Therefore, in this study, the scavenging activity of *C. odonthphyllum* fruit was also evaluated for OH radicals. In this assay, the averaged value obtained for skin was 43.33 ± 13.85 mg DMSOE/mg, flesh was 7.812 ± 1.42 mg DMSOE/mg, and kernel was 3.31 ± 0.80 mg DMSOE/mg with the differences being significant ($p < 0.05$). The scavenging activity determined by this assay showed that the skin had the highest antioxidant activity compared to other parts.

The mechanism of spin-trapping in electron spin resonance (ESR) involved OH free radical scavenging activity by forming an adduct (Isao and Lawrence, 1990) which is similar with the mechanism of DPPH free radical scavenging activity. However, the OH radical assay offers an advantage over the DPPH radical assay as it overcomes the limitation occurring in the determination of scavenging activity using spectrophotometer in DPPH assay. In human tissues, it is believed that the production of hydroxyl radical takes place through Fenton reaction involving the reaction of ferrous ion and hydrogen peroxide (Halliwell and Gutteridge, 1986) as shown in (1). The formation of the hydroxyl radicals can be directly confirmed by the ESR spin-trapping technique. In this system, the presence of antioxidant can act as OH free radical trapper which formed the adduct as in (2), that detected by electromagnetic wave from the ESR machine. The more adduct produced in the system, indicates the higher antioxidant that is present to be the trapped towards the OH free radical (Isao and Lawrence, 1990).

$$Fe^{2+} + H_2O_2 \qquad Fe^{3+} + HO^- + HO^{\cdot} \quad .....(1)$$

$$AO + OH^{\cdot} \longrightarrow AO\text{-}OH \text{ (}\textit{adduct}\text{)} \quad .....(2)$$

AO = antioxidant; OH = hydroxyl free radical

Findings in the present study, showed that the adduct formed was highly concentrated in the skin extract of *C. odontophyllum*. The high concentration of adduct might be due to the presence of high antioxidant in the skin which is parallel with the high phenolic content in skin of the fruit (Table 1).

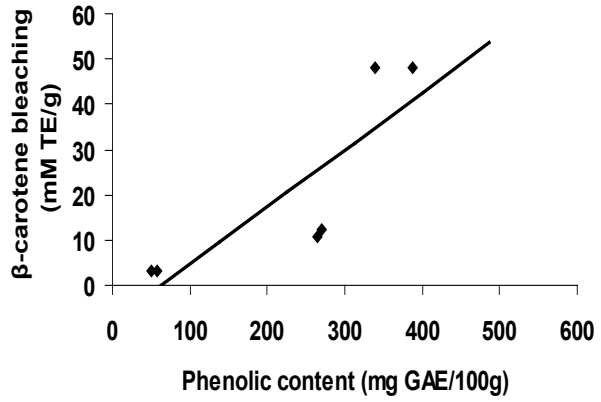

Figure 1. Linear correlation of β-carotene bleaching value with phenolic content of *Canarium odontophyllum.*

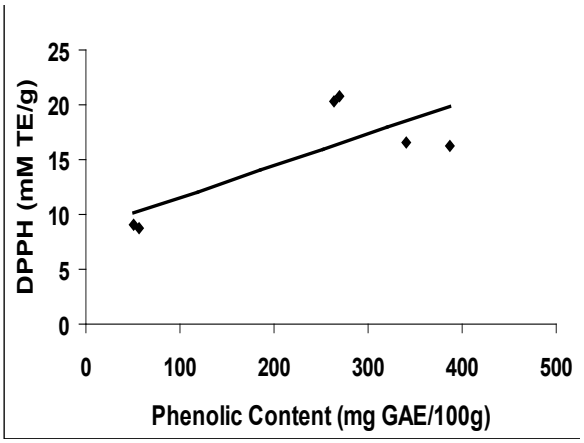

Figure 3. Linear correlation of DPPH value with phenolic content of *Canarium odontophyllum*

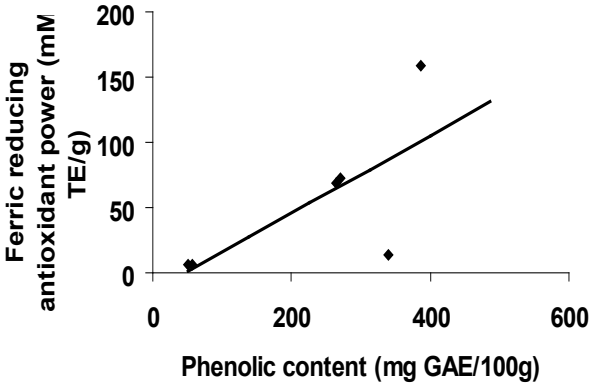

Figure 2. Linear correlation of FRAP value with phenolic content of *Canarium odontophyllum*

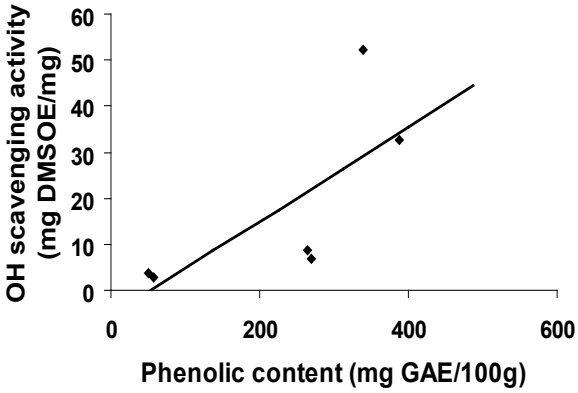

Figure 4. Linear correlation of OH scavenging activity value with phenolic content of *Canarium odontophyllum*

Table 1. Total phenolic content in skin, flesh, and kernel of *Canarium odontophyllum*

Fraction	Total phenolic content (mg GAE/100g dry weight)*
Skin	387.5 ± 33.23[a]
Flesh	267.0 ± 4.24[b]
Kernel	51.0 ± 0.00[c]

* Values are the mean ± standard deviation (n=3)
[a, b, c] Different superscripts in the same column indicate the significant difference ($p< 0.05$)

Total phenolics content

Table 1 shows the total phenolic content in skin, flesh, and kernel of *Canarium odontophyllum*. The skins extract of *Canarium odontophyllum* possessed the highest phenolic content followed by flesh and kernel. There were significant differences between these values at $p < 0.05$. The differences in the phenolic content might be due to the variation of phenolic compounds in each fraction of the fruit.

The phenolic compounds, mainly characterized as phenolic acid and flavonoids have always been related to the antioxidant activity (Kähkönen *et al,* 1999). Phenolic acid is a natural antioxidant found in fruits and vegetables. The role of phenolic compounds as scavengers of free radical is emphasized in several reports (Madsen and Bertelsen, 1995; Moller *et al.,* 1999). Results obtained in the present study exhibited that *Canarium odontophyllum* is a potential source for natural antioxidant. The mechanism involved in Folin-Ciocalteu method however, is based on the reducing power of phenolic hydroxyl groups which is not very specific but detects all phenols, non-phenolics and reducing sugar with varying sensitivity. A reduction-oxidation reaction of phenolase occurs under alkaline conditions reducing the phosphotungstic-phosphomolybdic complex in the reagent to a blue color (Waterman and Mole, 1994; Milardovic *et al.,* 2006).

Correlation between total phenolic content and antioxidant assays

There were high correlations between total phenolic content and all antioxidant activity assays using Pearson correlation. β-carotene bleaching activity, reducing power and scavenging activity of the fruit fractions showed high correlation with total phenolic content with *r*-values of 0.84 (Figure 1), 0.71 (Figure 2), 0.78 (Figure 3) and 0.74 (Figure 4), respectively. The results showed that phenolic compounds could be the important antioxidant in the fractions of the fruit.

High correlation between total phenolic content and antioxidant capacity has been observed in plum fruits (Gil *et al.,* 2002; Kim *et al.,* 2003a; Kim *et al.,* 2003b; Chun and Kim, 2004). Most of the phenolic compounds especially anthocyanins and neochlorogenic acid are concentrated in the skin of plum and other fruits (Raynal *et al.,* 1989). Study on cherry fruits also showed that phenolic compounds were concentrated in skin of dark colored fruits (Vangdal and Slimestad, 2006). Based on this characteristic, the purplish blue skin of *Canarium odontophyllum* might contain high level of phenolic compounds and potentially be used for various medicinal purposes.

Conclusion

This study indicated that methanolic extracts of skin, flesh, and kernel of *Canarium odontophyllum* possessed antioxidant properties. Antioxidant properties of various *Canarium odontophyllum* extracts may offer wide application for medicinal purposes as well as functional properties in foods. Therefore, the information gathered may be of great interest in both academia and the food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones.

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