Antioxidative Phytochemicals and Anti-Cholinesterase Activity of Native Kembayau (Canarium Odontophyllum) Fruit of Sabah, Malaysian Borneo

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Abstract

Canarium odontophyllum, known locally as “Kembayau” or “Dabai”, is a highly seasonal fruit which is popular among local people in Borneo Island. This study was conducted to determine the antioxidant activity, phytochemicals (total phenolic, total flavonoid, total anthocyanin and total carotenoid contents) and acetylcholinesterase inhibitor potential of extracts of the flesh and seed of this fruit. C. odontophyllum was collected in Beaufort, Sabah, Malaysian Borneo and subsequently freeze-dried and extracted using 80% methanol and distilled water. Antioxidant activities were analyzed using DPPH free radical scavenging, ABTS decolorization and FRAP (Ferric reducing / antioxidant power) assays. Anti-Alzheimer’s potential was determined using acetylcholinesterase enzyme inhibition assay. The results showed that the total phenolic and flavonoid contents were higher in the flesh of C. odontophyllum with the values of 11.96 ± 0.05 mg gallic acid equivalent (GAE)/g and 10.11 ± 1.54 mg rutin equivalent (RU)/g, respectively. Total anthocyanin and carotenoid content were also higher in the flesh of the fruit with the values of 12.75 ± 0.28 mg/100g and 2.84 ± 0.11 mg/100g. The flesh of the fruit also showed higher antioxidant activity as assessed using DPPH, ABTS and FRAP assays. However, anti-cholinesterase activity was higher in the seed of C. odontophyllum which showed that other phytochemical content (besides phenolic and flavonoid) might be responsible to the observed effects. The same trend of phytochemicals, antioxidant and anti-cholinesterase activity was also observed in the distilled water extract. These findings suggested that C. odontophyllum is not only nutritious but also displayed potential pharmacological properties.

Keywords: Canarium odontophyllum; Phytochemicals; Antioxidants; Acetylcholinesterase inhibition activity

Introduction

There are increasing researches that focus on phytochemical compounds which display diverse pharmacological properties [1]. Since synthetic antioxidants such as butylated hydroxytoluene (BHT) have been shown to be carcinogenic, natural antioxidants from plants and vegetables are considered to be preferable [2]. Consumption of fruits and vegetables has been shown to lower the risk and incidence of certain types of cancer, cardiovascular diseases, and diabetes [3]. In addition, previous study has shown the ability of fruits and vegetables to act as anti-Alzheimer’s agents [4].

The rich biodiversity in Borneo offers many edible underutilised fruits that are yet to be fully explored. One such underutilized fruit is Canarium odontophyllum (commonly called “Kembayau” in Sabah, Malaysia and “Dabai” in Sarawak, Malaysia), which contains many important nutrients and is rich with phytochemicals that have potential health benefits [5,6]. The unripe skin of the fruit is light yellow in colour and turns to dark purple/black when ripe (Figure 1). The flesh and the skin of C. odontophyllum are edible. The flesh is white-yellow in colour and the texture is hard when raw. Local people usually eat the fruit by pouring hot water into the fruits for 10 to 15 minutes to soften the flesh.

The flesh of the fruit of C. odontophyllum weighs about 7-10 g which constitutes almost 47% of the total weight; the seed of the fruit contribute the remainder of the fruit. The diameter of the fruit is about 7-10 cm, and has a hard single three-angled seed (Figure 2). The fruit is

![Figure 1: The ripe fruit of Canarium odontophyllum.](image1)

![Figure 2: The three-angled seed of Canarium odontophyllum.](image2)
The fruit of *C. odontophyllum* was collected from Beaufort, Sabah, Malaysia during October to December 2011. The fruit was cleaned, weighed and separated into edible (flesh+skin) and inedible (seed) components. Small cut pieces were freeze-dried, and the freeze-dried samples were ground into fine powder using a dry grinder. The ground samples were sieved to get uniform size, then kept in an air-tight container and stored in a freezer (-20°C) until further analysis.

Samples (0.1 g) were extracted for 2 h with 80% methanol or distilled water with a ratio of 1:50 at room temperature on an orbital shaker set at 200 rpm [12]. The mixture was then centrifuged at 1400g for 20 min and the supernatant decanted into a 15ml vial. The pellet was re-extracted under identical conditions. The supernatants were combined and used for analysis of total antioxidant activity, total phenolic, total flavonoid, total anthocyanin contents and total carotenoid content, together with antioxidant and anti-cholinesterase activity.

**Determination of total phenolic content**

Total phenolic content was determined using Folin-Ciocalteu reagent as adapted from Velioglu et al. [12] with slight modifications. Extract (300 µl) was mixed with 2.25 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min. To this was added 2.25 ml of sodium carbonate (60 g/l) solution. After 90 min at room temperature, absorbance was measured at 725 nm using spectrophotometer. Standards of gallic acid in the concentration ranges from 0 to 100 µg/ml were run with the test samples, from which a standard curve was plotted. The results were expressed as mg gallic acid equivalents in 1 g of dried sample (mg GAE/g).

**Determination of total flavonoid content**

Total flavonoid content was determined by using colorimetric method described by Dewanto et al. [13] with slight modification. Briefly, 0.5 ml of extract was mixed with 2.25 ml of distilled water in a test tube followed by addition of 0.15 ml of 5% NaNO₂ solution. After 6 min, 0.3 ml of 10% AlCl₃·6H₂O solution was added, and allowed to stand for another 5 min before 1.0 ml of 1 M NaOH was added and allowed to stand for another 5 min. The mixture was mixed well with vortex, and the absorbance measured immediately at 510 nm using spectrophotometer. Standards of rutin in the concentration ranges from 0 to 100 µg/ml were run with the test samples, from which a standard curve was plotted. The results were expressed as mg rutin equivalents in 1 g of dried sample (mg RE/g).

**Materials and Methods**

**Plant materials and extraction**

In terms of health, *C. odontophyllum* benefits humans with its high antioxidant capacity that is mainly contributed to the total phenolic compound in the fruits [7]. In addition, the fruit is also nutritious due to the high content of protein, carbohydrate and fat. Besides, it is also rich in minerals such as potassium, phosphorus, calcium and magnesium [6]. A previous study showed that other *Canarium* species have potent antimicrobial activity [8]. He and Xia [9] reported that some *Canarium* species are rich in oleic and linoleic acids which are beneficial to skin as they can reduce acne, act as anti-inflammatory agent as well as maintaining the moisture of the skin [10]. Koudou et al. [11] reported that the essential oil of other *Canarium* species can also act as a painkiller. In light of the promising activity of *Canarium* species, this study was conducted to investigate the phytochemicals, antioxidant and anti-cholinesterase potential of *C. odontophyllum* fruit collected in Sabah, Malaysian Borneo.

**Determination of total anthocyanin content**

Total anthocyanin content was measured using a spectrophotometric pH differential protocol described by Giusti and Wrolstad [14] with slight modification. Briefly, 0.5 ml of the extract was mixed thoroughly with 3.5 ml 0.025 M potassium chloride buffer pH 1. The mixture was mixed with vortex and allowed to stand for 15 min. The absorbance was then measured at 515 and 700 nm against distilled water blank. The extract was then combined similarly with 0.025 M sodium acetate buffer pH 4.5 and the absorbance was measured at the same wavelength after being allowed to stand for 15 min. The total anthocyanin content was calculated using the following equation:

\[
\text{Total anthocyanin content (mg/100 g of dried sample) = } A \times \text{MW} \times DF \times 1000 / (c \times C) \]

where A is absorbance=(A₅₃₅–A₇₅₀) pH 1.0–(A₅₃₅–A₇₅₀) pH 4.5; MW is molecular weight for cyanidin-3-glucoside=449.2; DF is the dilution factor of the samples, c is the molar absorbtivity of cyanidin-3-glucoside=26900; C is the concentration of the buffer in mg/mL. The results were expressed as mg of cyanidin-3-glucoside equivalents in 100 g of dried sample (mg c-3-gE/100 g dried sample).

**Determination of total carotenoid content**

Total carotenoid content was measured by using the method of Hess et al. [15] with slight modification. In brief, 300 µl sample was added to 300 µl distilled water. The mixture was mixed with 1.2 ml n-hexane of the hexane layer. The mixture was centrifuged for 5 minutes at 4°C and absorbance was measured at 350 nm in a spectrophotometer. Standards of β-carotene in the concentration ranges from 0 to 100 µg/ml were run with the test samples, from which a standard curve was plotted. The results were expressed as mg of β-carotene in 100 g of dried sample (mg BC/100 g dried sample).

**DPPH free-radical scavenging assay**

The scavenging activity of the extract was measured by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a free radical model and a method adapted from Magalhaes et al. [16]. An aliquot (300 µl) of samples or control (80% methanol or distilled water) was mixed with 3.0 ml of 500 µM (DPPH) in absolute ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min in the dark. Absorbance of the mixture was measured spectrophotometrically at 517 nm, and the free-radical scavenging activity was calculated as follows:

\[
\text{Scavenging effect (%)} = \left[ 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right] \times 100
\]

**FRAP (Ferric reducing/antioxidant power) assay**

This procedure was conducted according to Benzie and Strain [17] with slight modification. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM FeCl₃·6H₂O in a 10:1:1 ratio prior to use and heated to 37°C in a water bath. A total of 3.0 ml FRAP reagent was added to a test tube and a blank reading was taken at 593 nm using spectrophotometer. A total of 100 µl of selected plant extracts and 300 µl of 0.1 M FeCl₃·6H₂O solution were added to the test tubes, respectively. After 30 min, the mixture was mixed well with vortex, and the absorbance measured immediately at 593 nm using spectrophotometer. Standards of ascorbic acid in the concentration ranges from 0 to 100 µg/ml were run with the test samples, from which a standard curve was plotted. The results were expressed as mg ascorbic acid equivalents in 1 g of dried sample (mg AAE/g).
μl of distilled water were added to the cuvette. A second reading at 593 nm was performed after 90 min of incubation at 37°C in water bath right after the addition of the sample to the FRAP reagent. The changes in absorbance after 90 min from initial blank reading were compared with standard curve derived from standard of Fe (II) concentrations in the range 0 and 1000 µg/ml. A standard curve was then plotted. The final result was expressed as the concentration of antioxidant having a ferric reducing ability in 1 gram of sample (µM/g).

**Results and Discussion**

**Total phenolic content**

The reduction of phosphomolybic acid to molybdenum blue by the hydroxyl (-OH) groups of phenolic compounds in the presence of an alkaline medium is the principle used in this method. The blue coloured complex can be detected spectrophotometrically at a wavelength of 760 nm [20]. The total phenolic content of C. odontophyllum was higher in the flesh than in the seed (Table 1). The results of this study are in line with those of previous studies which also reported that the edible parts of C. odontophyllum (i.e peel and pulp) exhibit high phenolic content as compared to the seed part of the fruit [21,22]. Ellagic acid and vanillic acid are among the phenolic acids that are reported to be present in C. odontophyllum [23]. High polarity of organic solvent in 80% methanolic extract proved to be more efficient to extract phenolic compounds in the sample rather than mono component solvent, thus explaining the reason of why methanolic extract yields higher phenolic content than in aqueous extract in the present study [24].

**Total flavonoid content**

A previous study has demonstrated that the fruits of C. odontophyllum contain flavonoids, namely catechin and epicatechin. These flavonoids might contribute to the antioxidant activity in the fruit [23]. The present study showed that the total flavonoid content is higher in the flesh of C. odontophyllum compared to the seed part in both 80% methanol and aqueous extract (Table 1). Prasad et al. [21] also reported higher flavonoid content in both peel and pulp of C. odontophyllum rather than seed when extracted with ethyl acetate, n-butanol and water which is also similar to the present study. Among the two extracts, the 80% methanol extract always showed higher flavonoid content than the aqueous extract. This is in keeping with the observation that a mixture of two solvents producing a more polar medium facilitates extraction of more phytochemical compound [24].

**Total anthocyanin content**

Monomeric anthocyanins undergo a reversible structural transformation as a function of pH where the coloured oxonium form exists at pH 1.0 and the colourless hemiketal form exists at pH 4.5. Thus, the difference in absorbance of the pigment at 520 nm is proportional to the concentration of pigment [25]. Anthocyanins are usually associated with the colour of the fruits, being particularly responsible for the pink, red, blue and purple colours in plants [26]. Higher anthocyanin content was detected in the flesh part of C. odontophyllum as compared to its seed part (Table 1). The results of this study are in line with those of previous studies which also reported that the edible parts of C. odontophyllum (i.e peel and pulp) exhibit higher flavonoid content than the seed part of the fruit [21,22].

**Statistical analyses**

All experiments were carried out in 3 replicates in 3 independent experiments. The results were presented as mean ± standard deviation (SD) using Prism 5 software. The data were statistically analysed by one-way ANOVA and Duncan post-hoc test, with a level of statistical significance set at p ≤ 0.05. Correlation analysis was performed by Pearson’s Test.

**Table 1**: Content of total phenolics, total flavonoids, total anthocyanins and total carotenoids in extracts of fruit of C. odontophyllum.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Phenolics1 (mg GAE/g)</th>
<th>Total Flavonoids2 (mg RE/g)</th>
<th>Total Anthocyanins3 (mg C-3-GE/100g)</th>
<th>Total Carotenoids4 (mg BC/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% methanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flesh</td>
<td>11.96 ± 0.05†</td>
<td>10.11 ± 1.54†</td>
<td>12.75 ± 0.28†</td>
<td>2.84 ± 0.11†</td>
</tr>
<tr>
<td>Seed</td>
<td>5.54 ± 0.19†</td>
<td>3.84 ± 0.05†</td>
<td>0.82 ± 0.01†</td>
<td>0.66 ± 0.09†</td>
</tr>
<tr>
<td>Aqueous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flesh</td>
<td>5.72 ± 0.10†</td>
<td>5.04 ± 0.19†</td>
<td>9.06 ± 0.03†</td>
<td>1.56 ± 0.12†</td>
</tr>
<tr>
<td>Seed</td>
<td>3.94 ± 0.12†</td>
<td>2.78 ± 0.06†</td>
<td>0.53 ± 0.07†</td>
<td>0.33 ± 0.03†</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD (n = 3) which, with different letters (within column), are significantly different at p < 0.05.

1Total phenolic content was expressed as mg gallic acid equivalents in 1 g of dried sample (mg GAE/g).
2Total flavonoid content was expressed as mg rutin equivalents in 1 g of dried sample (mg RE/g).
3Total anthocyanin content was expressed as mg of cyanidin-3-glucoside equivalents in 100 g of dried sample (mg C-3-GE/100 g dried sample).
4Total carotenoid content was expressed as mg of β-carotene in 100 g of dried sample (mg BC/100 g dried sample).
seed in both extracts (Table 1). The flesh part of *C. odontophyllum* which is dark purple in colour might be contributed mostly by the anthocyanin as suggested in a previous study [23].

**Total carotenoid content**

Epidemiological studies show that carotenoid-rich foods protect against cancer, heart disease and diabetes [27]. The present study indicated higher total carotenoid content in the flesh part of *C. odontophyllum* as compared to the seed part. Carotenoid synonymously known to gives colour to the fruits. Hence, this is clearly explained why the flesh (outer part) have more carotenoid contents as compared to its seed (Table 1). Previously, carotenoids profiles of peel, pulp and seed of the fruit and their related antioxidant capacities showed that the peel and pulp was enriched with all-trans-β-carotene, when compared with the seed part of *C. odontophyllum* [28], which also supported the findings of the present study.

**Scavenging activity on 2,2-diphenyl-2-picrylhydrazyl radical**

The process of either electron or hydrogen donation cause the reduction of purple colour of DPPH [29]. The ability of the antioxidant to decolorize the purple DPPH colour indicates the ability of the sample to act as donor to the DPPH free radicals [30]. The scavenging activity of the sample towards the free radicals can be measured spectrophotometrically at 518 nm.

In both extracts, the flesh of *C. odontophyllum* displayed higher scavenging effects as compared to the seed parts (Table 2). There were significant differences on the scavenging free radical activity in the flesh and seed part for both extracts (p<0.05). DPPH free radical scavenging activity has been reported to show high correlation with inhibition capacity towards lipid peroxidation process [31]. Higher scavenging activity in flesh of *C. odontophyllum* was also reported in a previous study by Azrina et al. [22], whilst Wei et al. [32] reported that *Canarium album Raesusch* fruit displayed stronger DPPH radical-scavenging ability than ascorbic acid.

**Ferric reduction based on FRAP assay**

Ferric Reducing Antioxidant Power (FRAP) method is one assay to determine the antioxidant capacity in samples which utilise Single Electron Transfer mechanism. In this study, the Fe(III)-tripyridyltriazine (TPTZ) complex is reduced to the Fe(II)-TPTZ-complex by an antioxidant sample, this latter complex possessing an intense colour in an acidic environment [17].

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH Assay (%)</th>
<th>FRAP Assay (mM Fe(II)/Liter)</th>
<th>ABTS Assay (mg AEAC/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flesh</td>
<td>88.14 ± 1.42</td>
<td>30.52 ± 0.54</td>
<td>46.71 ± 0.98</td>
</tr>
<tr>
<td>Seed</td>
<td>80.84 ± 0.76</td>
<td>17.00 ± 0.79</td>
<td>41.52 ± 0.36</td>
</tr>
<tr>
<td>Aqueous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flesh</td>
<td>77.50 ± 0.36</td>
<td>26.43 ± 0.76</td>
<td>43.29 ± 0.89</td>
</tr>
<tr>
<td>Seed</td>
<td>73.39 ± 0.15</td>
<td>13.52 ± 0.21</td>
<td>30.38 ± 0.08</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD (n = 3) which, with different letters (within column), are significantly different at P<0.05.

1DPPH free radical scavenging activity was expressed as % of scavenging.

2FRAP was expressed as µM ferric reduction to ferrous in 1 g of dry sample.

3ABTS scavenging activity than the seed part.

**Figure 3**: The anti-cholinesterase activity (%) of different parts of 80% methanol and aqueous extracts of the fruit of *C. odontophyllum* (n=3) (*p* significant at *p* < 0.05 as compared to the aqueous value at the same concentration).

The FRAP activity of the fruit of *C. odontophyllum* was higher in the flesh compared to the seed. The same trend was also observed in the aqueous extract (Table 2). This finding was similar to that of a previous study by Prasad et al. [21] which demonstrated that peel exhibited higher reducing power as compared to the seed. Azrina et al. [22] also reported higher reducing ability of the skin and flesh of *C. odontophyllum* as compared to the seed part. The flesh part of *C. odontophyllum* have acceptable reducing power which might act as primary and secondary antioxidant [33].

**ABTS scavenging assay**

The principle of ABTS decolorization assay is quite similar to that of the DPPH assay in that it involves the scavenging activity of extracts against free radicals, but ABTS salt must be generated by enzymatic or chemical reaction [34]. For both extracts, the higher scavenging activity was found in the flesh part of the fruit of *C. odontophyllum* (Table 2). These findings support those of Prasad et al. [28] which also demonstrated the skin and pulp of the *C. odontophyllum* to have higher ABTS scavenging activity than the seed part.

**Anti-cholinesterase activity**

Alzheimer's disease is a form of dementia characterized by loss of central cholinergic neurons associated with a marked reduction in content of acetylcholinesterase (AChE), the enzyme responsible for the termination of nerve impulse transmission at cholinergic synapses. Consequently, one therapeutic approach to treatment of Alzheimer's disease is use of anti-cholinesterase drugs. Measurement of AChE activity is based on the yellow colour produced from thiocholine (product from acetylthiocholine) when it reacts with dithiobisnitrobenzoate ion [35,36]. The ability of the plant extracts to inhibit AChE can then be determined. The present study showed that only the 80% methanol extracts of the flesh and seed of *C. odontophyllum* displayed anti-cholinesterase activity when tested at 0-100 µg/ml (Figure 3). The 80% methanol extract inhibited AChE activity in a concentration-dependent manner such that a concentration of 100 µg/ml, the flesh and seed extracts displayed anti-cholinesterase activity of 22.4% and 18.6%, respectively. However, distilled water extracts of the flesh and seed of *C. odontophyllum* showed no or low anti-cholinesterase activity (less than 1%) as compared to the 80% methanol extracts. A previous study by Mogana et al. [37] showed the presence of scopoletin compound isolated in the leaves part of *Canarium patentinervium* Miq. (Burseraseae Kunth.) which inhibits the acetylcholinesterase enzyme moderately.
Relation between phytochemicals contents, antioxidant activity and anticholinesterase activity

Correlation analysis was performed to investigate the correlation between phytochemicals and antioxidant activity as well as antioxidant activity with anti-cholinesterase activity. The results demonstrated a strong positive correlation between the antioxidants assays with the total phenolics, flavonoids and carotenoids contents across the samples tested. The FRAP assay data displayed positive correlation with the phenolic, flavonoid, anthocyanin and carotenoid content with the r values of 0.827, 0.887, 0.987 and 0.962 respectively, while the ABTS data and the total phenolic, flavonoid, anthocyanin and carotenoid content also showed strong positive correlation with the r values of 0.756, 0.773, 0.778 and 0.818 respectively. The DPPH data also showed positive correlation with phenolic, flavonoid and carotenoid content with r values of 0.944, 0.909 and 0.818 with only a moderate positive correlation with anthocyanin content (r=0.693).

The results of this study were in agreement with earlier literature by Kim et al. [38] which showed that the phenolic content in the plum showed positive correlation with antioxidant activity of the samples. Anthocyanins, which are suspected to be concentrated in the purplish-blue peel might also contribute to the antioxidant activity in the fruit of *C. odontophyllum* [22].

The anti-cholinesterase activity also showed moderately positive correlation with antioxidant activity in the samples by FRAP and ABTS assays with r values of 0.403 and 0.639 respectively. Meanwhile, anti-cholinesterase inhibition activity in the present study showed a strongly positive correlation with DPPH assay with an r value of 0.913. Previous work by Amoo et al. [39] indicated positive relationship between antioxidant activity and AChE inhibitory properties of some selected long-term stored African medicinal plants, whilst the study by Jo et al. [40] also reported positive correlation between acetylcholinesterase and antioxidant activity of seed and pericarp of *Camelina sinensis* L.

Conclusion

The current study provides evidence that the flesh of the fruit of *C. odontophyllum* has potential as a natural antioxidant and an acetylcholinesterase inhibition agent with high content of phenolics, flavonoids, anthocyanins and carotenoids. Besides nutritional benefits, *C. odontophyllum* also displays pharmacological properties which might offer potent application in food and pharmaceuticals formulations.

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References


