

Evaluation of Antioxidant activities, Anthocyanins, Total Phenolic Content, Vitamin C Content and Cytotoxicity of *Carissa carandas* Linn.

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ABSTRACT

Carissa carandas, commonly known in Thai as ‘nham-daeng’, has a long history in alternative medicine in Thailand. Ethanolic extracts of fresh leaves, unripe fruits and fully-ripe fruits were investigated for their antioxidant activity, total phenolic content, total anthocyanins, anthocyanin identification, vitamin C content and cytotoxicity. Results showed that the leaf extract exhibited the highest antioxidant activity, FRAP value and total phenolic content. The antioxidant activities of leaf, unripe and fully-ripe fruit extracts were 65.24 ± 0.69 , 0.85 ± 0.03 and 2.42 ± 0.41 mg AAE/g, respectively. Total phenolic contents were 24.91 ± 0.22 , 1.29 ± 0.16 and 4.67 ± 0.41 mg GAE/g and total anthocyanins were 0, 0.33 ± 0.04 and 54.80 ± 6.07 mg/l, respectively. The fully-ripe fruit extract, which showed low antioxidant activity and total phenolic contents, exhibited the highest total anthocyanins and cyanidin-3-galactoside levels. The unripe fruit extract showed the highest content of vitamin C (300.75 ± 57 mg/100g). The major anthocyanin of all samples was cyanidin-3-galactoside. At a concentration of 200 μ g/ml, treatment with the leaf extract caused the greatest reduction in HepG2 viability among the three extracts. These results indicated that the amounts of total phenolics and antioxidant activity could be correlated with cytotoxicity on HepG2 cells for the leaf sample.

Keywords: *Carissa carandas*, antioxidant, anthocyanin, cytotoxicity

INTRODUCTION

Carissa carandas Linn. is a tropical tree that belongs to the Apocyanaceae family. Its botanical name is *Carissa congesta* Wight, but the name *C. carandas* is still widely used in the literature. The plant is commonly known as karanda (India and Malaysia), nham-daeng (Thailand), caramba (Philippines) and ci huangguo (Chinese). The plant is native to India and cultivated in Taiwan, Indonesia, Malaysia, Burma, Sri Lanka, Thailand and the Pacific Islands (Wiert, 2006). *C. carandas* is a large dichotomously branched evergreen shrub with short stem and strong thorns in pairs (Hegde et al, 2009). Whole plant and its parts are used in tradition-

al medicine for treatments of ailments. Unripe fruits could be used to treat liver dysfunction, break fever and counteract putrefaction of blood (Wiert, 2006). They have also been studied for antidiabetic, analgesic and anti-inflammatory properties (Itankar et al., 2011; Begum et al., 2013). Ripened fruits were taken as an antiscorbutic and a remedy for biliousness. The roots contain salicylic acid and cardiac glycosides, which could cause a slight decrease in blood pressure (Devmurari et al., 2009). They also contain carissone and methyl ester, which contributed to anti-diarrheal and antianthelmintic properties. The leaf extracts were shown to have cytotoxic effects towards HeLa cells (human cervical cancer cells), PC-3 cells (human prostate cancer cells) and 3T3 cells (normal mouse fibroblasts) (Begum et al., 2013). The chemical constituents of leaves include carandiol (Begum et al., 2013), betulinic acid (Sharma et al., 2010), β -sitosterol-3-0- β -D-glucopyranoside (Son et al., 1998), oleanolic acid (Seebacher et al., 2003), ursolic acid (Seebacher et al., 2003) and 4-hydroxybenzoic acid (Pandey et al., 2011). The fruits and leaves appear rich in tannins (Morton, 1987). Considering the above information, the present study aimed to evaluate the antioxidant activity, total phenolic content, total anthocyanin content, anthocyanin identification, vitamin C content and cytotoxicity of *C. carandas* from Thailand.

MATERIALS AND METHODS

Sample preparation

Unripe fruits (white in color), fully-ripe fruits (dark purple in color) and leaves were collected from Samut Songkhram Province, Thailand. Ten grams of samples were soaked in 100 ml of 40% ethanol in water for 24 hours. The extracts were filtered through No. 4 Whatman filter papers.

Free radical-scavenging assay with DPPH

The antioxidant activity was performed as previously described by Khattak et al. (2008) with slight modifications. It was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. About 100 μ l of each extract was added to 900 μ l of DPPH in methanol solution (150 μ M) in a test tube and shaken vigorously. After incubation at room temperature for 15 min in darkness, the absorbance of each solution was determined at 517 nm. The free radical-scavenging activity was expressed as ascorbic acid equivalent.

Ferric reducing antioxidant potential (FRAP)

FRAP assay was performed according to the method described by Benzei and Strain (1996). The FRAP reagent was prepared by mixing 16.7 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 8.3 mM 2,4,6-tripyridyl-s-triazine (TPTZ) with 250 mM acetate buffer, pH 3.6. A total of 75 μ l sample and 225 μ l of distilled water were added to 2.25 ml of freshly prepared FRAP reagent in a test tube. The mixture was incubated at room temperature throughout the reaction. The absorbance was read at 596 nm using a spectrophotometer immediately and 30 min after mixing. The antioxidant potential of the samples was analyzed based on a calibration curve plotted using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at concentrations ranging from 400 to 2,000 μ M.

Total phenolic content

The content of reducing components was estimated using the Folin-Ciocalteu assay according to the method developed by Velioglu et al. (1998). First, 0.75 ml of 10-fold diluted Folin-Ciocalteu reagent and 100 μ l of the ethanolic extract were placed in a test tube. The mixture was mixed and allowed to stand at room temperature for 5 min. Then, 0.75 ml of 6% (w/v) sodium carbonate solution was added. The mixture was homogenized and allowed to stand at room temperature for 90 min. Total phenolic content was determined using a spectrophotometer at 725 nm. The standard calibration curve was plotted using ascorbic acid at the concentration of 0.02-0.1 mg/ml. The total phenolic content was expressed as gallic acid equivalent (GAE) mg/g.

Total anthocyanin content

Total anthocyanin content was measured by the pH-differential spectrophotometric method (Wrolstad et al., 2005). Each sample was diluted to two dilutions with potassium chloride (0.025 M) at pH 1.0 and sodium acetate (0.4 M) at pH 4.5. They were allowed to equilibrate for 15 min before detection by spectrophotometer. The absorbance was measured at 520 nm and 700 nm. The difference in the absorbance at differing pH values and wavelengths was calculated as:

$$A = (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH}1.0} - (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH}4.5}$$

The concentration of total anthocyanin pigments was calculated as:

$$\text{Total anthocyanin content (mg/l)} = (A \times \text{MW} \times \text{DF} \times 1000) / \epsilon \times l$$

where MW is the molecular weight, DF is the dilution factor, ϵ is the molar absorptivity and l is for 1 cm path length. The molecular weight (MW = 449.2 g mol⁻¹) and the molar absorptivity ($\epsilon = 26,900 \text{ Lcm}^{-1}\text{mol}^{-1}$) of cyanidin-3-glucoside were used.

Identification of anthocyanins

Anthocyanins were identified using HPLC (Jasco, Japan) according to Lee et al. (2005). The flow rate was 1 ml/min and the injection volume was 20 μ l. The column temperature was maintained at 40°C. A Luna 5 μ m C18(2), 100°C (250 x 4.6 mm) column from Phenomenex was used (Torrance, CA). Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. The program used a linear gradient from 2 to 20% solvent A in 25 min, with detection at 520 nm. The content of individual anthocyanin was calculated based on the calibration curve for cyanidin-3-galactoside.

Vitamin C content

Ascorbic acid was analyzed by HPLC (Jasco, Japan) with a UV detector. The separation was carried out using a C18, Phenomenex column (250 mm x 4.6 mm i.d. and 5 μ m particle size) and the run time was 15 min. The ascorbic acid peak was detected at 205 nm. The entire chromatographic separation was performed at an isocratic mobile phase of methanol and 0.05 M potassium dihydrogen phosphate (pH 3.0) at the ratio 3:97 v/v.

Cytotoxicity test

In vitro cytotoxicity was measured using MTT assay according to Plumb et al. (1989). HepG2 cells were seeded at 4,000 cells/well and grown for 48 hours. Then, *C. carandas* extracts were added to the cells at specified concentrations in fresh media. After 24 hours, the media with the extracts were removed, and the cells were allowed to grow in fresh media for an additional 24 hours. Fifty μl of MTT in phosphate buffer at 5 mg/ml was added to the medium in each well and the cells were incubated for 4 hours. The medium with MTT was then aspirated from the wells, and formazan solubilized with 200 μl of dimethylsulfoxide and 25 μl of Sorensen's Glycine buffer, pH 10.5, was added. The optical density was read with a microplate reader at a wavelength of 570 nm.

Statistical analysis

Analysis of variance (ANOVA) compared the mean values. All statistical analyses were performed using SPSS software. All the assays were carried out in triplicate. The results are expressed as mean values with standard deviation (SD).

RESULTS

Antioxidant activities of *C. carandas*

The antioxidant activities present in 40% (v/v) aqueous ethanol extracts of leaf, unripe and fully-ripe fruit extracts are summarized in Table 1. The leaf extract showed the highest DPPH-scavenging activity (65.24 ± 0.69 mgAAE/g), followed by the fully-ripe fruit extract (2.42 ± 0.21 mgAAE/g) and the unripe fruit extract (0.85 ± 0.03 mgAAE/g). Similarly, the leaf extract exhibited the highest FRAP value of 400.84 ± 8.72 $\mu\text{molFeSO}_4/\text{g}$, whereas the values for both fruit extracts were much smaller; 37.81 ± 1.07 $\mu\text{molFeSO}_4/\text{g}$ for the fully-ripe fruit extract and 25.94 ± 1.90 $\mu\text{molFeSO}_4/\text{g}$ for the unripe fruit extract. No significant ($p > 0.05$) difference of unripe and fully-ripe fruits was found in antioxidant activity and FRAP value. The total amount of phenolic content present in aqueous ethanol extracts of *C. carandas* is shown in Table 1. The results were expressed as gallic acid equivalent (GAE/g). The leaf extract exhibited the highest total phenolic content 24.91 ± 0.22 , followed by the fully-ripe fruit extract 4.67 ± 0.59 and the unripe fruit 1.25 ± 0.16 mgGAE/g. ANOVA indicated that the means of antioxidant activities and total phenolics were significantly different ($p < 0.05$) between leaves and fruits samples. However, the unripe and the fully-ripe fruits did not show any significant differences.

Vitamin C content

The vitamin C content of *C. carandas* leaves and fruits are shown in Table 1. Unripe fruits showed a high vitamin C level of 300.75 ± 57.65 mg/100g while fully-ripe fruits and leaves gave lower vitamin C levels. The amounts of vitamin C in fully-ripe fruits and in leaves were 180.40 ± 43.09 mg/100g and 149.58 ± 17.42 mg/100g, respectively. Significant differences in vitamin C content were observed among unripe fruits, fully-ripe fruits and leaves.

Table 1. Antioxidant activity (DPPH assay), FRAP, total phenolic content, total anthocyanins and vitamin C content of *Carissa carandas*.

Samples	DPPH assay (mgAAE/g)	FRAP ($\mu\text{molFeSO}_4/\text{g}$)	Total phenolic content (mgGAE/g)	Total anthocyanin content ¹ (mg/l)	Vitamin c (mg/100g)
Leaves	65.24±0.69 ^a	400.84±8.72 ^a	24.91±0.22 ^a	0.00 ^b	149.58±17.42 ^b
Unripe fruits	0.85±0.03 ^b	25.94±1.90 ^b	1.25±0.16 ^b	0.33±0.04 ^b	300.75±57.65 ^a
Fully-ripe fruits	2.42±0.21 ^b	37.81±1.07 ^b	4.67±0.59 ^b	54.80±6.07 ^a	180.40±43.09 ^b

Note: Values are expressed as mean ± SD of triplicate measurements. Different letters in the same column indicate significant differences at $p < 0.05$. GAE = gallic acid equivalent; AAE = ascorbic acid equivalent. ¹Total anthocyanin content was expressed in terms of cyanidin-3-glucoside.

Total anthocyanin content

The total anthocyanin content of the aqueous ethanol extract of *C. carandas*, determined by the pH differential method, was 54.80±6.07 mg/l for fully-ripe fruits and 0.33±0.04 mg/l for unripe fruits, whereas in leaf samples, anthocyanins could not be detected. The fully-ripe fruits were dark purple in color and exhibited higher antioxidant activity, higher total phenolic contents and higher total anthocyanin contents than unripe fruits that were white in color. The anthocyanin composition of *C. carandas* fruits was determined with HPLC analysis. Identification and peak assignment of anthocyanins were primarily based on their retention time comparing to that of the standard compounds. *C. carandas* fruits were shown to contain cyanidin-3-galactoside (Figure 1).

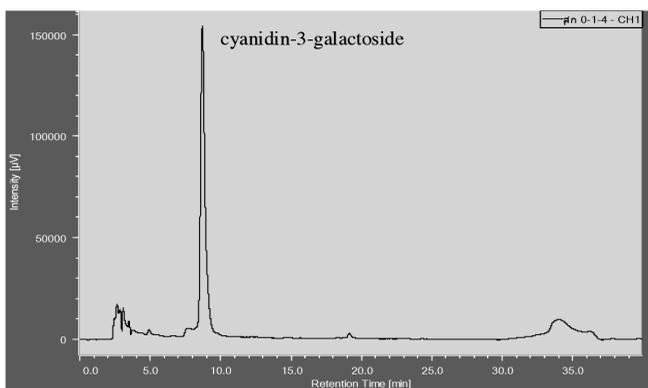


Figure 1. HPLC chromatogram of anthocyanin in the ethanolic extract of *Carissa carandas*.

Cytotoxicity test

The aqueous ethanol extracts of *C. carandas* were evaluated for their cytotoxic property on cultured human hepatocellular carcinoma HepG2 cells (Figure 2). At concentrations up to 100 $\mu\text{g/ml}$, no significant effects on cell viability were found for all extracts. Significant reduction in viability was seen at 200 $\mu\text{g/ml}$ of the extracts ($p < 0.05$). It appeared that the leaf extract was most effective; HepG2 viability was reduced to 67±8% at this concentration.

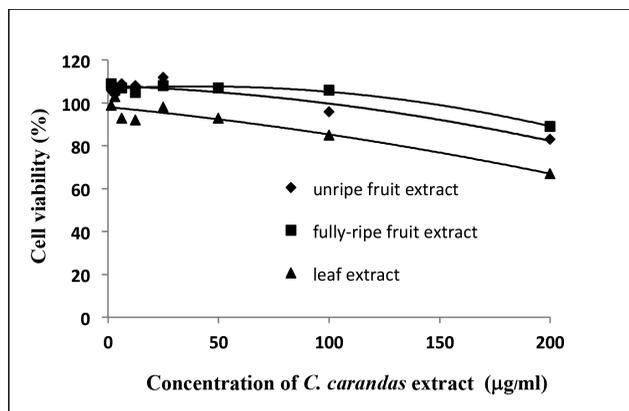


Figure 2. Cytotoxic effects of *Carissa carandas* on human hepatocellular carcinoma cells (HepG2).

DISCUSSION

The antioxidant activity (DPPH assay), FRAP, total phenolic content, total anthocyanin content and vitamin C content of *Carissa carandas* were evaluated in this study. The resulting antioxidant activity could be related to the total phenolic content. The ethanolic leaf extract had the highest antioxidant activity, which correlated with its total phenolics results. The results of our study were also comparable with those reported by many researchers. The antioxidant activity and the total phenolics of leaf samples were found to be similar to those of the leaf extract of *Etilingera* species (Zingiberaceae) and *Macaranga* species (Chan et al., 2007; Lim et al., 2009). Leaves of *C. carandas* possessed a high total phenolic content when compared to the values in other tropical medicinal plants. For example, Lim and Murtijaya (2007) reported lower values of the total phenolic content (13.00–16.00 mgGAE/g) for leaf extracts of *Phyllanthus amarus*. The high antioxidant activity of *C. carandas* was probably due to high levels of polyphenolic compounds, such as phenolic acids and flavonoids (Guo et al., 1997). Phenolics, such as flavonoids, phenolic acids and tannins, are considered the major contributors of antioxidant activities in fruits and vegetables (Meng et al., 2011). However, our finding showed a lower value of the total phenolic content than that of *Carissa opaca* leaves (78.9±1.70 mgGAE/g in methanol) (Sahreem et al., 2010). The differences in the total phenolic content could be attributed to several factors, including extraction methods and solvents used, parts and kinds of plant utilized, the geographical origin and the cultivar used (Van der Sluis et al., 2001; Khattak et al., 2008).

In addition, our results supported the report of Brown et al. (2005); phenolic acids and anthocyanins contributed to a higher antioxidant activity. The fully-ripe fruit extract had a total phenolic content similar to the results obtained from *Carissa opaca* and *Mangifera indica* fruits (Ikhrum et al., 2009; Sahreem et al., 2010). Anthocyanins that were present in fully-ripe fruits likely contributed to the higher antioxidant activity (Kahkonen et al., 1999). Moreover, Wang et al. (1997)

reported that colored fruits, such as grapes and blueberries, contained high levels of anthocyanins, which yielded a high antioxidant activity. These observations indicated that fully-ripe fruits of *C. carandas* could be a good source of anthocyanin pigments. The major anthocyanin observed in this study was cyanidin-3-galactoside. This finding was in contrast to the report by Francis (1989), which stated that cyanidin-3-glucoside was the most common anthocyanin pigment in nature. The observed difference might be due to the extraction process and the nature of the plant itself.

For the cytotoxicity test, the leaf extract was most effective; HepG2 viability was reduced to $67\pm 8\%$ at the concentration of 200 $\mu\text{g/ml}$. These findings agreed with literature data. *C. carandas* leaf extract was shown to inhibit the proliferation of human cervical cancer cells (HeLa), prostate cancer cells (PC-3) and normal mouse fibroblasts (3T3) (Begum et al., 2013). Hence, further studies will be conducted to determine the response of HepG2, as well as other cell types, to higher concentrations of the extracts.

CONCLUSION

The ethanolic leaf extract of *C. carandas* exhibited the highest total phenolic content, followed by the extracts of fully-ripe and unripe fruits. Similarly, the antioxidant activity and FRAP values were highest in the leaf extract, correlating well with its total phenolic content. Moreover, the leaf extract was most effective in reducing the viability of HepG2 cells, and the significant effect could be seen starting at 200 $\mu\text{g/ml}$. Fully-ripe fruits showed the highest anthocyanin content, which contributed to their total phenolic content. Even though unripe fruits exhibited the lowest antioxidant activity, FRAP and total phenolic content, it contained the highest level of vitamin C. It can be concluded that *C. carandas* leaves can be used as an easily accessible source of natural antioxidants. In addition, fully-ripe fruits are a good source of anthocyanin, whereas, unripe fruits are a good source of vitamin C.

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