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Study of free radical scavenging, total phenolic contents and Tyrosinase inhibition activity of crude extract from *Carissa carandas* Linn.

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Abstract

The present study investigated the changes in total phenolics contents (TPC), tyrosinase inhibition and antioxidant capacity of *Carissa carandas* Linn. extracts. The effects of different solvent extraction (70% ethanol and 100% ethanol) on the phenolic profile of *Carissa carandas* Linn. fruit and their tyrosinase inhibition and antioxidant activity were studied. The result showed that the amount of crude extracts from 70% ethanol and ethanol was 5.76 and 2.54% respectively. In fact, the 70% ethanol extract showed the highest content of phenolic compounds at 28.21 mg GAE g^{-1} crude extract. Results indicated that the ethanol extract exhibited higher DPPH free radical scavenging activity than 70% ethanol extract. ABTS radical scavenging activity and ferric reducing power of 70% ethanol extract showed the highest antioxidant activities of 82.54% and 3494.80 mg g^{-1} crude extract, respectively. The percentage of tyrosinase inhibition was examined dopachrome method, the 70% ethanol extract exhibited the higher tyrosinase inhibition at 77.65%. The preliminary findings reveal that 70% ethanol extract has a high potential to be used as a natural skin whitening agent in cosmetic products.

Keywords: Phenolic contents; antioxidant activity; tyrosinase inhibition

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1. Introduction

Nowadays, the development of herbal cosmetic products such as whitening and anti-aging have focused on the plant extracts due to growing of consumer demands for healthy cosmetic from natural sources [1]. Several vegetables, particularly medicinal plants, have been widely studied for their natural antioxidant and tyrosinase inhibition. *Carissa carandas* Linn. is one of Thai traditional plants which possess antioxidant activity due to high amount of vitamin C and anthocyanin in the fruit. It is not only used for food, but also for other purposes, including medicine, anti-inflammatory and antioxidant activity herb. The high total phenolic content of the crude extract of this ripe fruit was reported at 7.80% by J. Sueprasarn, *et al*, in 2013. Moreover, the ripe fruit of this plant has been reported that contained anthocyanin to get high antioxidant properties. Additionally, the content and composition of phenolic compounds such as isoamyl alcohol, benzyl acetate, lupeol, oxalic acid, tartaric acid, citric acid, malic acid, malonic acid and glycolic acids of the ripe fruit had been reported. [2]. The Carandas plant, raw fruits and ripe fruits were showed in Fig. 1.

Fig. 1 (a) Carandas plant (b) raw fruits and (c) ripe fruits

It is well known that phenolic and flavonoid contents are important antioxidant substances obtained from most natural plants. These substances are able to reduce free radicals like superoxide, peroxyl, alkoxylate and hydroxyl. Thailand is one of important center origins for the genus *Carandas* Linn., and some important *Carandas* genotypes are originated from there. Up till now, the information about the antioxidant compounds in different fruit tissues, especially those of the Thai native *Carandas* genotypes, and their antioxidant capacity is still limited. The aims of this study are to evaluate the total phenolic content, tyrosinase inhibition and antioxidant capacity of different solvent crude extracts from the ripe fruit tissues of *Carissa carandas* Linn. and to identify the relationship between the antioxidant capacity evaluated by DPPH and ABTS free radical methods and the total phenolic contents. The results will provide important information for the future study and use *Carandas* genetic resources fully. Chemical constituents of *Carissa carandas* Linn. were depicted in Fig. 2.

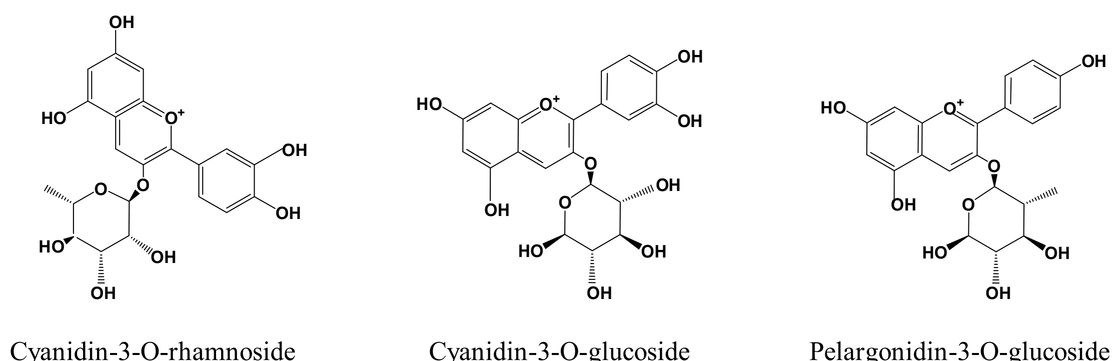


Fig. 2 Chemical constituents of *Carissa carandas* Linn.

2. Materials and methods

Reagents and standards

1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Butylated hydroxytoluene (BHT), ascorbic acid, ammonium molybdate, sodium phosphate, sulphuric acid, gallic acid, $FeCl_3$, $K_3Fe(CN)_6$, Sodium carbonate and Folin-Ciocalteu reagent (FCR) were purchased from Merck (Darmstadt, Germany). All the other chemicals and solvents used were of analytical grade.

Plants materials and preparation of extracts

The fruits of *Carissa carandas* Linn. were washed with water and air dried at room temperature. After drying, the seed was separated and grinded to small pieces. Phenolic compounds were extracted by maceration at room temperature using absolute ethanol and 70% ethanol/water (70:30 v v^{-1}) as solvent. The extraction was performed at the ratio of 1 g of the fruits to 2 ml of the solvent for 7 days. The extraction was done in triplicate. Then the collected extract was concentrated in vacuum at 45 – 50 °C to obtain a dry extract and stored at 4°C for further use [3]. The extraction method was described in Fig. 3.

(a) Washing and air dried (b) separated of the seed (c) solvent extractions (d) crude extracts

Fig. 3 The extraction method of Carandas ripe fruits.

DPPH scavenging activity

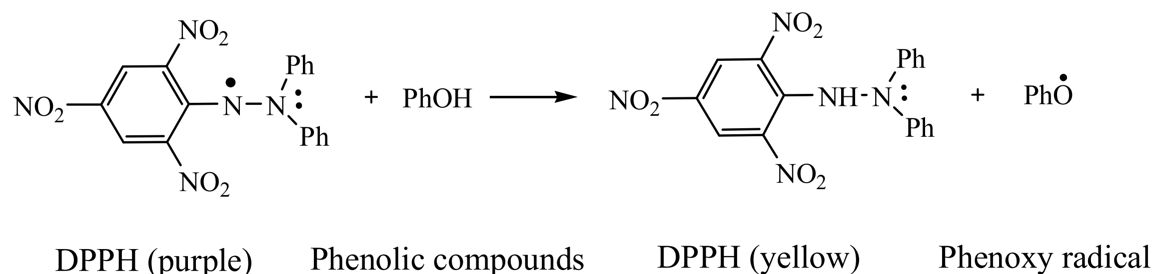
The free radical scavenging activity of each extract solution on DPPH radical was determined as described previously by Choompujun et al. [4]. The ethanol and 70% ethanol extract solution were prepared at concentration of 15,000 ppm by dissolving the crude extract 0.375 g in 10% H_2O:ethanol 25 mL [5]. In each tube, 2 mL of the sample solution was allowed to react with 2 mL of 1 mM of DPPH solution. The solutions were shaked and incubated at room temperature for 30 min in the dark. The absorbance of the resulting solution was measured at 515 nm with a spectrophotometer (Spectronic genesis 5). The DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH scavenging effect } (\%) \text{ or } \% \text{ inhibition} = \frac{(A_0 - A_1) \times 100}{A_0} \quad (1)$$

A_0 = The absorbance of the blank (methanol+DPPH) at 30 minutes
A_1 = The absorbance of the extracts (extract+DPPH) at 30 minutes

The mechanism of DPPH free radical scavenging activity was evaluated in Fig. 4.

Step 1



Step 2

Fig. 4 DPPH free radical scavenging mechanism of phenolic compounds.

Ascorbic acid with the concentration of 6.25 – 500 mg L^{-1} was used as reference standard compound and the experiment was done in triplicate. The concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against the extract concentration [6].

Ferric reducing antioxidant power assay (FRAP)

The ability of extracts to reduce iron (III) was determined according to the method of Choompujun et al. [4] with slight modifications. Both extracts were prepared at the concentration of 5000 ppm, and then 0.30 mL of the extract solution was mixed with 0.3 mL of $FeSO_4.7H_2O$ standard solution, 0.90 mL of FRAP reagent and 0.09 mL of distilled water in the test tube [4]. The mixture was allowed to react for 8 min at room temperature. The absorbance of the samples was measured at 539 nm. High absorbance of the reaction mixture indicated high reducing power. The absorbance of $FeSO_4.7H_2O$ standard solution with the concentration of 50 – 750 µmol was used for standard curve.

ABTS radical scavenging activity

The ABTS radical scavenging activity of the fruits extracts was carried out following the procedure of Re et al. [7] with a slight modification [8]. The extract solutions were prepared at concentration of 15000 ppm by dissolving the crude extract 0.125 g in 25 mL of 10% H_2O : ethanol. The 0.10 mL of ethanol extract solution was added to 3 mL of $ABTS^{+\cdot}$ reagent 4 ml. The mixture was incubated at room temperature for 6 min. The absorbance of the reaction was measured at 734 nm. The ABTS radical scavenging potential of the sample extracts was calculated using the following equation:

$$\%\text{ABTS radical scavenging activity} = \frac{(A_0 - A_1) \times 100}{100} \qquad (2)$$

A_0 = The absorbance of the blank (ethanol+ABTS) at 6 minutes
A_1 = The absorbance of the sample extracts (extract+ ABTS) at 6 minutes

The experiment were performed in triplicate.

Determination of total phenolic compounds content (TPC)

The total phenolic content was determined using the Folin-Ciocalteu colorimetric method with minor modification [2]. The ethanol and 70% ethanol extract solutions were prepared at concentration of 20000 ppm by dissolving the crude extract 0.5 g in 10% H_2O : ethanol 25 mL. Then 1.25 mL of the extract solution was allowed to react with 1.25 mL of Folin-Ciocalteu reagent in 25 mL volumetric flask. The solutions were shaked and incubated at room temperature for 5 min. Next 5 mL of 10% (w v^{-1}) sodium carbonate and 17.50 mL of distilled water were added to the mixture and then the flask was thoroughly shaken. After 10 minutes of reaction at ambient temperature, the absorbance measurements were recorded at 730 nm. The same procedure was repeated for the standard gallic acid solution.

The concentration of total phenolic contents in the extracts was expressed as mg of gallic acid equivalent pergram of sample (mg GAE g^{-1} dry extract).

Anti-tyrosinase assay

Tyrosinase inhibition assay was performed by using the modified dopachrome method with L-DOPA as substrate [9]. Briefly, the extracts and compounds were dissolved as following the method in Table 1. The reaction was carried out using 96-well and ELIS microplate reader to measure the absorbance at 490 nm. The mixtures were incubated at 37 ºC for 10 min, then 20 µL of L-DOPA and 40µL of L-tyrosine were put in each well. The reaction mixture was incubated at 25 ºC for 10 minutes and the absorbance was measured at 490 nm.

Vitamin C was used as reference standard inhibitors for comparison. The percentage of tyrosinase inhibition (I%) was calculate as follows:

$$I\% = (A - B) - (C - D)/(A - B) \times 100 \quad (3)$$

Where A is the absorbance of the control reaction, B is the absorbance of the blank control, C is the absorbance of the test samples and D is the absorbance of the blank sample.

Table 1 The extracts and compounds dissolved in 96-well plate.

	0.20 M Phosphate buffer pH 6.8	10% H_2O : EtOH	Test sample	Tyrosinase solution (203.30 units/M)	L-DOPA
A (Control)	140 µL	20 µL	-	20 µL	20 µL
B (Blank control)	160 µL	20 µL	-	-	20 µL
C (Test sample)	140 µL	-	20 µL	20 µL	20 µL
D (Blank sample)	160 µL	-	20 µL	-	20 µL

Statistical analysis

Data obtained from antioxidant activity, total phenolic compounds, and tyrosinase inhibition results were stated in mean ± SD of triplicate measurements. Analysis were performed by statistical analysis system. Data were considered statistically different at $P < 0.01$.

3. Results and Discussion

Extraction yield

According to solvent extraction, type of solvent is the important factors that affected the extraction yield. 100% Ethanol and 70% ethanol extraction process gave red viscous crude extract with the extraction yield of 5.76% and 2.54% respectively.

DPPH scavenging activity

In this study, the crude extracts from difference extraction solvents were investigated for their antioxidant activity with DPPH scavenging activity [5] as showed in Table 2. Results indicate that the ethanol extract showed higher antioxidant activity which gave inhibition of 80.75% comparable to the 70% ethanol extract of 72.37% (P<0.01).

Table 2 DPPH scavenging activity of difference extracts from *Carissa carandas Linn.* fruits.

Concentration of the extract	**%DPPH scavenging activity**	
	100% Ethanol extract	**70% Ethanol extract**
mean	$80.75^{a} \pm 0.02$	$72.37^{a} \pm 0.03$

[a] (P < 0.01)

Determination of total antioxidant capacity, ABTS

The 70% ethanol crude extract of *Carissa carandas* Linn. fruits showed significantly higher ABTS radical scavenging activity than that of ethanol crude extract (P<0.01) [8]. The two difference extraction solvents had different levels of antioxidant capacity. The crude extract of 70% ethanol solvent gave ABTS radical scavenging activity of 82.54% and the crude extract of ethanol solvent of 61.72%. Similar results were found by Chandra et al. in which the *Carissa carandas* Linn. fruits showed good antioxidant activity and contained high phenolic compounds of 7.80% of crude extract.

Table 3 ABTS radical scavenging activity of difference extracts from *Carissa carandas* Linn. fruits.

Concentration of the extract	%ABTS radical scavenging activity	
	Ethanol extract	70% Ethanol extract
mean	$61.72^{a} \pm 0.00$	$82.54^{a} \pm 0.087$

[a] (P < 0.01)

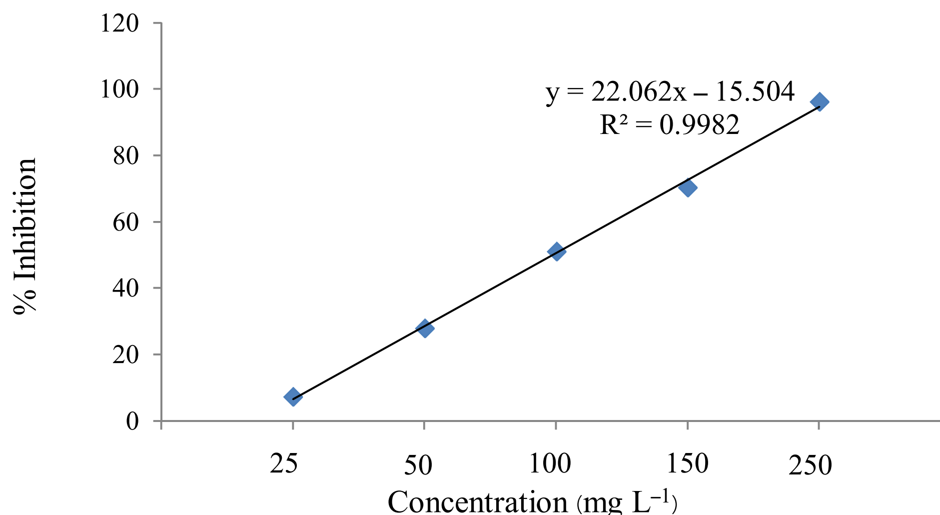


Fig. 5 ABTS radical scavenging activity of Vitamin C (%Inhibition)

Ferric Reducing antioxidant power (FRAB) determination

Generally, the reducing properties associated with the presence of compounds which exerted their action by breaking the free radical chain via donating a hydrogen atom . In the ferric to ferrous iron reduction assay [4], the electron donation capacity of the extracts was assessed and compared to that of ferrous sulfate in Fig. 6. The reducing powers of the crude extracts from *Carissa carandas* Linn. fruits are showed in Table 4. Significant difference was observed between 70%ethanol extract 3494.804 Fe^{2+} g^{-1} extract and ethanol extract 2085.230 Fe^{2+} g^{-1} extract ($P < 0.01$).

Table 4 Ferric Reducing antioxidant power of difference extracts from *Carissa carandas* Linn. fruits.

Concentration of the extract	Fe^{2+}TPTZ (mg g^{-1} extract)	
	Ethanol extract	70% Ethanol extract
mean	2,085.23[a]	3,494.80[a]

[a]($P < 0.01$)

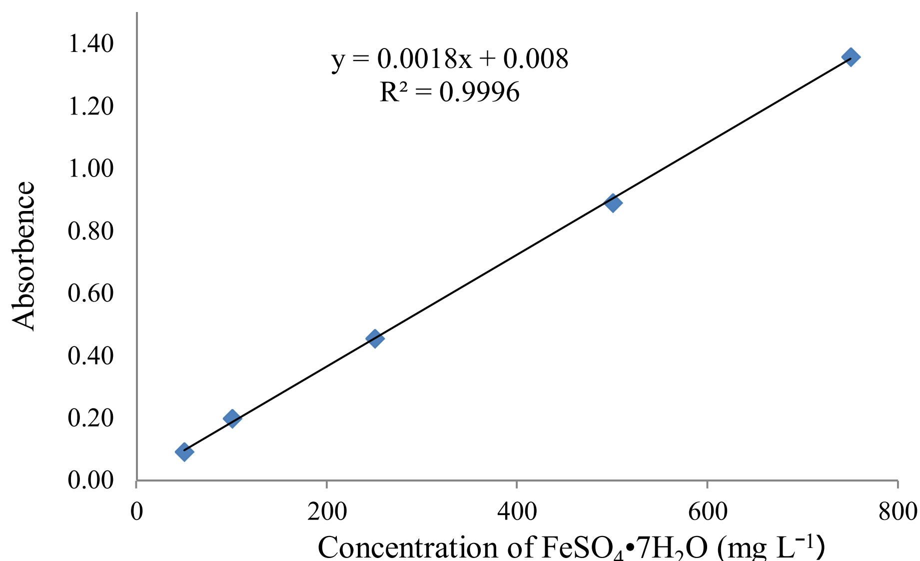


Fig. 6 Standard curve of $FeSO_4.7H_2O$ solution.

Total phenolic contents

The average of total phenolic contents of crude extracts from *Carissa carandas Linn.* fruits tested of each solvent are presented in Table 5 [2]. The results showed that the extraction solvent significantly influenced of the total phenolic contents ($P < 0.01$). In fact, 70% ethanol extract had the higher total phenolic contents of 28.21 mg GAE g^{-1} extract, while ethanol extract represents lower content of 20.02 mg GAE g^{-1} extract.

Table 5 Total phenolic contents of difference extracts from *Carissa carandas* Linn. fruits.

Concentration of the extract	Total phenolic contents (mg GAE g^{-1} extract)	
	Ethanol extract	70% Ethanol extract
mean	20.20[a]	28.21[a]

[a](P < 0.01)

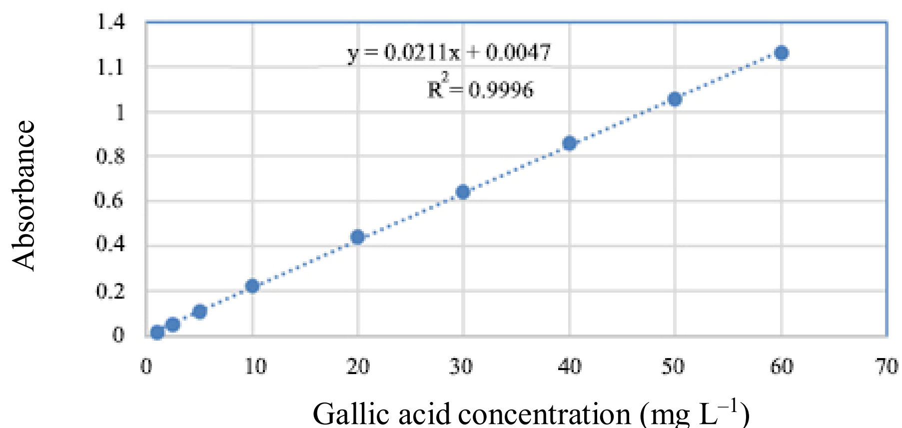


Fig. 7 Standard curve of gallic acid at the absorbance 730 nm.

Tyrosinase inhibition

The solvent response studies have indicated a different potent inhibition of the enzyme tyrosinase [9]. The 70% ethanol extract gave higher percentage of inhibitory activity against tyrosinase at 77.65% than that of ethanol extract at 71.84% (P < 0.01) showed in Table 6.

Table 6 Tyrosinase inhibition of difference extracts from *Carissa carandas* Linn. fruits.

Concentration of the extract	% tyrosinase inhibition	
	Ethanol extract	70% Ethanol extract
mean	71.84[a]	77.65[a]

[a](P < 0.01)

4. Conclusion

In order to show the antioxidant potentials of *Carissa carandas Linn.* fruits and to ascertain the difference solvent extraction in their chemical constituents, the present work was the comprehensive study of total phenolic contents, antioxidant activity and tyrosinase inhibition of the *Carissa carandas Linn.* It is extremely important to point out that, there was a correlation between antioxidant activity potential and amount of phenolic compounds in both extracts from the difference solvent extraction, in agreement with the previous investigation. The phenolic content estimated in our results was probably responsible for the total antioxidant capacity and reducing power of the *Carissa carandas Linn.* fruit. Phenolic compounds were important components which could be used for the free radical-scavenging activity.

Comparing the plant from the two difference solvent extraction, we found that ABTS radical scavenging activity, reducing power assays, total phenolic contents and tyrosinase inhibition of 70% ethanol extract were 82.54, 3494.80 mg g^{-1} extract, 28.21 mg GAE g^{-1} extract and 77.65 which showed a high degree of correlation and higher than that of ethanol extract. These results showed that the total phenolic contents have an obvious variation according to the different solvent extraction. The higher total phenolic contents have been considered powerful antioxidants and proved to be more potent antioxidants. The *Carissa carandas* Linn. fruits are good sources of phenols and natural antioxidants that might have benefits for health and suitable processed to herbal cosmetic. The preliminary findings reveal that 70% ethanol extract has a high potential to be used as a natural skin whitening agent in cosmetic products. However, ethanol extract gave higher DPPH scavenging activity at 80.75.

A number of authors reported high correlation between total phenolic contents and antioxidant capacity assays. These results suggest that the total phenolic contents are the key contributors to the antioxidant capacity. Solvent extraction of herbs has been found to be useful technique for increasing the amount of phenolic compounds and antioxidant capacity of the herb samples.

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