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A Green Method for Preparation of Cyanidin-3-glucoside from *Carissa carandas* Fruits and α-Glucosidase Inhibitory Activity Evaluation

Kittituch Saengkhaw[1], Nuttapong Arthan[1] and Pharkphoom Panichayupakaranant[1,2,*]

[1]*Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90112, Thailand*

[2]*Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90112, Thailand*

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*Corresponding Author

Tel: +66-74-288980
E-mail: pharkphoom.p@psu.ac.th

Abstract:

In this research, a tailored approach for the preparation of a cyanidin-3-glucoside-enriched extract (C3GE) and cyanidin-3-glucoside (C3G) from the fruits of *Carissa carandas* L. was achieved using a green methodology. The method involved a cold extraction, followed by fractionation processes on a hydrophobic (Diaion® HP-20) column using a hydroethanolic solvent system for column elution. C3GE was produced after the one-step fractionation, while C3G was obtained after the two-step fractionation. Based on an HPLC method, C3GE contained 34.8% w/w of C3G, while C3G was identified via its ^{1}H and ^{13}C NMR data. An *in vitro* assay for the α-glucosidase inhibitory effect revealed that C3GE and C3G possessed good inhibitory activity against α-glucosidase, with IC_{50} values of 19.7 and 4.4 μg/mL, respectively, which is better than that of acarbose (IC_{50} of 395.4 μg/mL). Our findings suggest the potential use of this green extraction method for the production of C3G and C3GE, as well as its application in functional ingredient industries, including nutraceuticals and pharmaceuticals.

1. INTRODUCTION

Carissa carandas (carandas plum or karonda) is a plant in the family Apocynaceae. Its edible fruits have been used by people to make various health products, such as jam, jelly, cookies, drinks, and beverages. It has been reported that ~~a~~ the fruit extract of *C. carandas* exhibited antioxidative, anti-inflammatory, and antidiabetic activities, both *in vitro* and *in vivo* studies [1-4]. The ripened fruits contain plenty of anthocyanins, of which the most abundant is cyanidin-3-glucoside (C3G) [5].

C3G is a well-known bioactive compound that contributes various health benefits, especially in non-communicable diseases, such as diabetes, cancer, cardiovascular diseases, and their complications [6]. Regarding antidiabetic properties, it has been reported that C3G inhibited α-glucosidase and dipeptidyl peptidase-4 and enhanced insulin sensitivity as well as glucose consumption in *in vitro* and *in vivo* models [7-10]. Therefore, C3G is a promising therapeutic agent, warranting further practical pharmaceutical and nutraceutical applications through its insightful extraction and purification methods.

Previous studies have reported that C3G could be obtained from various fruits containing red and blue pigments, such as blue honeysuckle (*Lonicera caerulea*), blood fruits (*Haematocarpus validus*), red raspberry (*Rubus ideaus*), and mulberry (*Morus alba*). However, the reported extraction and purification methods typically require a significant amount of toxic organic solvents, such as hexane, *n*-*tert*-butyl methyl ether, *n*-butanol, methanol, and acetonitrile, and often involve multiple steps [11-14]. Therefore, this research evaluated the potential of *C. carandas* fruits as an alternative source of C3G extract. Moreover, a simple green extraction and purification method using ethanol and water as green solvents for the whole process has been successfully established. This method is safe, simple, and low-cost, and can be practically applied for industrial productions.

2. MATERIALS AND METHODS

2.1. Plant Material

The ripened fruits of *C. carandas* were purchased from a local market in Hat-Yai, Songkhla, Thailand, in July 2022. The plant was identified by Professor Pharkphoom Panichayupakaranant and its voucher specimens (No. SKP 013 03 03 01) were kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand. The fruits were washed and cut, and the seeds were removed. The plant material was kept frozen at -20 °C until further use.

2.2. Chemicals

C3G (98% purity) was purchased from ChemFaces® (Wuhan, China). Ethanol phosphoric acid, HPLC grade acetonitrile, and dimethylsulfoxide were obtained from RCI Labscan, Thailand. Diaion® HP-20, acarbose, α-glucosidase (from *Saccharomyces cerevisiae*), and *p*-nitrophenyl-α-D-glucopyranoside were procured from Sigma-Aldrich (MO, USA).

2.3. Extraction

The frozen fruit (1 kg) was blended with ethanol (1 L) using a blender, set it aside for 1 h, and then filtered it through a filter paper. The fruit residue was extracted again with ethanol (500 mL) using the same method, and the pooled ethanol extracts were subjected to further fractionation processes.

2.4. Fractionation and Purification

Fractionation of the ethanol extract was carried out on a hydrophobic Diaion® HP-20 column (5 cm × 60 cm column containing 100 g hydrophobic resin) in order to produce a C3G-enriched extract (C3GE). The ethanol extract (400 mL) was loaded into the column and then eluted with a 30% v/v ethanol (1.6 L). Eight fractions of each 200 mL were collected. The pooled dark red color fractions 4 and 5 were dried using a rotary evaporator (Heidolph Hei-VAP Ultimate Control, Germany) at 50°C to obtain a dark red semisolid of C3GE.

C3GE was further purified on the same Diaion® HP-20 column but gradient eluted with 400 mL of 10% v/v, 20% v/v, and 30% v/v ethanol, respectively. The fractions with red color (80 mL) were collected and subjected to an HPLC determination of C3G. Based on the HPLC method, fraction 7 was subjected to evaporation to produce a dark red powder of C3G.

2.5. Identification of C3G

Identification of the obtained C3G was based on HPLC determination and it was compared with the authentic compound from ChemFaces®. The chemical structure (Fig. **1**) was confirmed by analyzing the ^{1}H NMR and ^{13}C NMR spectral data using a Bruker Avance Neo NMR spectrometer (Fällanden, Switzerland) at 500 and

125 MHz, respectively, and the data was then compared with the previously reported spectral data [11]. The NMR data of the obtained C3G are illustrated in Table **1**.

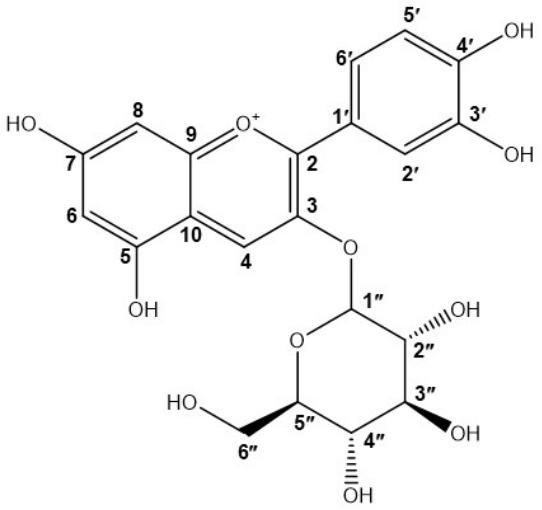

Figure 1: Chemical structure of C3G.

2.6. HPLC Method for Quantitative Determination of C3G

HPLC conditions for quantitative analysis of C3G in C3GE are composed of a 5 μm, 4.6 mm × 250 mm TSK-gel ODS-80Tm column (Tosho Bioscience, Japan) connected to a Shimadzu model with autosampler and a photodiode-array detector (Shimadzu Corp. Kyoto, Japan) and eluted with a gradient solvent system of 5% v/v to 100% v/v acetonitrile (in 0.05% phosphoric acid) for 10 min followed by 100% v/v acetonitrile for further 13 min, at a flow rate of 1 mL/min. The quantitative determination was carried out at 520 nm. The experiment was performed in triplicate. A calibration curve of C3G was established using five concentrations of C3G standard solutions (25, 50, 100, 200, and 400 μg/mL) to produce a linear regression equation of Y = 29674X - 39489 (r^2 = 1.0000).

2.7. *In Vitro* Assay for α-Glucosidase Inhibitory Activity

An *in vitro* assay against α-glucosidase activity was carried out by following a method described by

Table 1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of C3G (in CD_3OD)

Position	^{1}H NMR, δ (ppm)	^{13}C NMR, δ (ppm)
2	-	153.2
3	-	151.7
4	8.9 (s, 1H)	131.9
5	-	153.2
6	6.54 (d, 1H)	101.3
7	-	157.1
8	6.59 (d, 1H)	98.2
9	-	153.1
10	-	114.2
1′	-	122.5
2′	8.01 (d, 1H)	118.3
3′	-	144.2
4′	-	151.8
5′	6.74 (d, 1H)	116.3
6′	8.19 (dd, 1H)	131.7
1″	4.66 (d, 1H)	101.9
2″	3.65 (m, 1H)	73.4
3″	3.65 (m, 1H)	75.3
4″	3.65 (m, 1H)	70.2
5″	3.89 (m, 1H)	75.6
6″	3.90 (m, 2H)	61.2

Suttithumsatid *et al*. [15]. Acarbose was utilized as a positive control. The experiments were carried out in triplicate.

2.8. Statistical Method

The obtained results were calculated as mean ± standard deviation (SD). The statistical significance was analysed using one-way analysis of variance (ANOVA), followed by Tukey's multiple range test. A significant difference was confirmed when the *p* value was less than 0.05.

3. RESULTS AND DISCUSSION

The present study established a cold method for extraction of C3G from *C. carandas* fruits using ethanol as the solvent. The method produced a dark purple semi-solid extract after solvent evaporation, with an approximate yield of 1.73% w/w based on the fresh weight of the fruits. The cold extraction method yielded a purple-red extract, whereas the heat extraction method yielded a reddish-brown extract (data not shown). This confirms that C3G, which has a red color, is unstable or decomposed during heat [6, 16]. Based on the polarity of C3G (Log P of 0.39), ethanol is a suitable solvent for its extraction. On the basis of an HPLC method, we found that the crude ethanol extract of *C. carandas* fruits obtained from the cold extraction method had a C3G content of 5.54 ± 0.08 % w/w of dried extract. The result suggests that *C. carandas* fruits are a promising source of C3G and suitable for industrial productions of C3GE and C3G.

Recent studies have accepted the preparation of herbal extracts in the form of 'active constituent-enriched extract' as a feasible strategy to produce herbal extracts with a low-cost production but high-therapeutic efficacy compared to their pure active compounds [17-19]. Therefore, we subjected the crude ethanol extract of *C. carandas* fruits to a fractionation process using a reusable Diaion® HP20 column to produce C3GE. The solution of crude extract obtained from the extraction step was directly loaded into the column without any evaporation step, and the column was eluted by a hydroethanolic solvent system to accumulate C3G in the pooled fractions. Solvent evaporation resulted in the formation of a dark red semi-solid of C3GE. Based on a HPLC method (Fig. **2**), C3GE contained a C3G

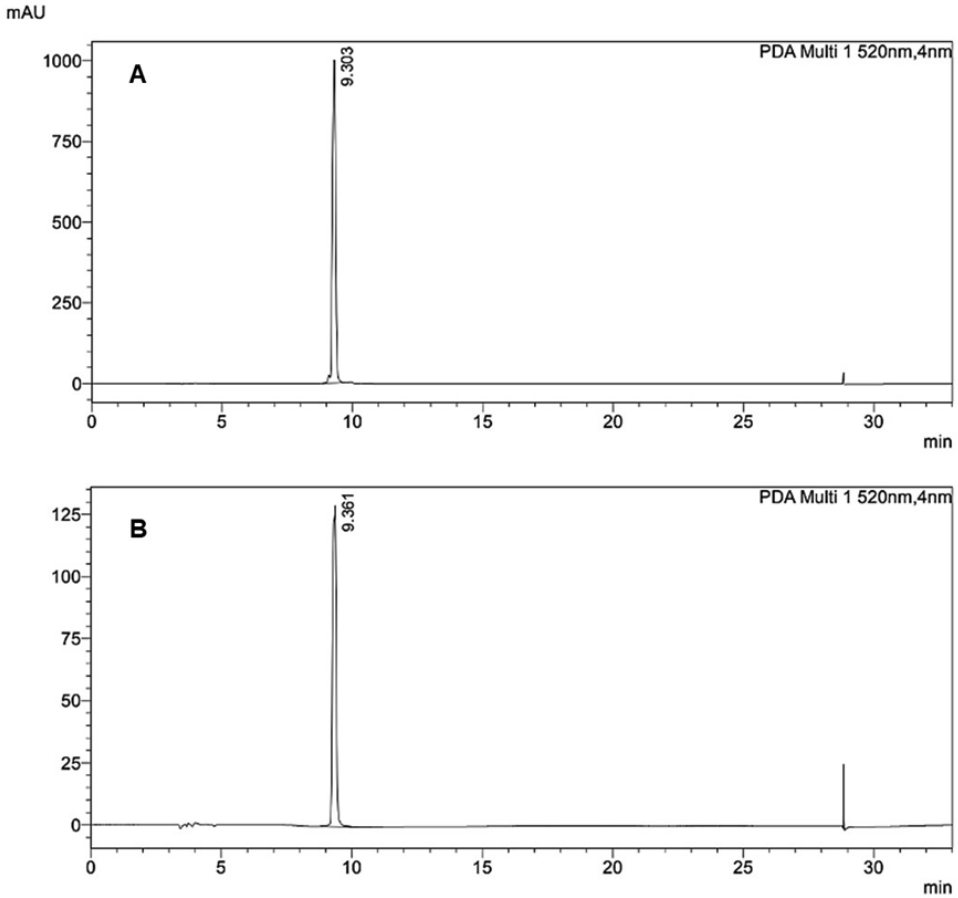

Figure 2: HPLC chromatograms of C3G (A) and C3GE (**B**).

content of 34.83 ± 0.51% w/w, which is approximately 6-times higher than that of the crude ethanol extract. Therefore, a simple fractionation step through the green chromatographic method is very useful to improve the C3G content and quality of the extract.

Furthermore, C3GE is a good resource for purifying C3G due to its high content. In addition, modifying the solvent system for column elution on a Diaion® HP-20 column appears to have potential for this purification. Therefore, C3GE was further purified on a Diaion® HP-20 column. We started the column eluting with 10% v/v ethanol to eliminate highly polar compounds like sugars and acids, and then gradually increased the ethanol concentrations up to 30% v/v to collect a fraction of C3G. Based on this chromatographic method, a purified dark red powder of C3G was obtained after solvent evaporation. The identification of C3G was then confirmed by comparing its HPLC chromatogram with the authentic compound as well as ^{1}H and ^{13}C NMR data with the previously reported data [11]. This purification method is safe, simple, and low-cost for purifying C3G.

It has been reported that C3G exhibited a good α-glucosidase inhibitory effect, both in *in silico* and *in vitro* studies [20]. To ensure the efficacy of C3GE, we further determined its *in vitro* inhibitory effect against α-glucosidase by comparing it with C3G and acarbose. The results revealed that C3GE exhibited a satisfactory α-glucosidase inhibitory effect with an IC_{50} value of 19.7 ± 0.62 µg/mL when compared to that of C3G (IC_{50} value of 4.4 ± 0.35 µg/mL) and acarbose (IC_{50} of 395.4 ± 12.23 µg/mL). Although C3GE possessed significantly lower activity than C3G, due to its three times lower content of C3G, the extract showed markedly and significantly higher activity than acarbose. This implies that nutraceutical and pharmaceutical applications could potentially utilize C3GE as a functional ingredient.

4. CONCLUSIONS

The green methods for preparations of C3GE and C3G established in this study are safe, simple, and low-cost, and can be practically applied for industrial productions. Our findings indicate that a cold extraction method combined with a Diaion® HP-20 chromatographic method may potentially be used for preparations of C3G and C3GE from the ripened fruits of *C. carandas*. In addition, C3GE can potentially be used as a functional ingredient in pharmaceutical and nutraceutical applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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