วารสารโรคมะเร็ง
THAI CANCER JOURNAL
ปีที่ 39 ฉบับที่ 1
มกราคม–มีนาคม 2562

Antioxidant and Antiproliferative Activities of *Carissa carandas* Linn. Fruits

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Abstract ***Carissa carandas*** **Linn. (family Apocynaceae), known in Thai as "Nam Daeng", has been recognized among some traditional medicine systems to cure various diseases, such as diarrhea and helminth infections, used as an analgesic, anti-inflammatory, anti-pyretic, and for liver diseases, rheumatism, and cancer. The purpose of this study was to determine the antioxidant and antiproliferative activities of *C. carandas* juice and methanolic extracts of *C. carandas*. Fresh ripe fruits of *C. carandas* were crushed and the juice extract was dried by lyophilizer. The residues of the crushed fruits were then macerated with methanol and the combined extracts evaporated to dryness. The juice and methanolic extracts were used for analysis. The antioxidative potentials of these extracts were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical ($OH^{\bullet}$) scavenging assays. Gallic acid and mannitol, antioxidant agents were used as positive controls. Antiproliferative activity was also analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay against human cancer cell lines; KB (human epidermoid carcinoma), MCF-7 (human breast adenocarcinoma), SiHa (human cervical carcinoma) and $HepG_2$ (human hepatocellular carcinoma) cell lines, as well as non-tumorigenic cells; Vero (African green monkey kidney) cell line. The results demonstrated that both juice and methanolic extracts of *C. carandas* fruits exhibited DPPH radical scavenging actions in a dose-dependent manner. Furthermore, the extracts showed hydroxyl radical scavenging activities. However, they showed weak antiproliferative activity toward any of the cells tested. It was concluded that *C. carandas* fruits possess antioxidative properties and may offer further application as chemopreventive agents.** *(Thai Cancer J 2019;39:6-15)*

Keywords: ***Carissa carandas*** **Linn., antioxidant activity, antiproliferative activity, cancer cell lines**

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ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งการเจริญของเซลล์ของสารสกัดจากผลหนามแดง

โดย **สุชานุช อ่อนดี**

งานเซลล์และภูมิบำบัด กลุ่มงานวิจัย สถาบันมะเร็งแห่งชาติ กรมการแพทย์ กระทรวงสาธารณสุข

บทคัดย่อ *Carissa carandas* Linn. หรือหนามแดง จัดอยู่ในวงศ์ Apocynaceae ถูกนำมาใช้เป็นยาสมุนไพรพื้นบ้านรักษาโรคต่างๆ เช่น โรคท้องร่วง ขับพยาธิ ระงับปวด ต้านอักเสบ แก้ไข้ รักษาโรคตับ โรคไขข้อ รวมทั้งโรคมะเร็ง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งการเจริญของเซลล์ของสารสกัดน้ำคั้นและสารสกัดเมทานอลจากผลหนามแดงสุก โดยการนำผลหนามแดงสุกมาบดและคั้นแยกน้ำออก น้ำคั้นจากผลหนามแดงสุกที่ได้ทำให้แห้งโดยวิธีการทำแห้งแบบแช่เยือกแข็ง (lyophilization) กากของผลหนามแดงที่เหลือจากการคั้นน้ำออกแล้วนำไปหมักด้วยตัวทำละลายเมทานอล ระเหยตัวทำละลายเมทานอลในสารสกัดที่ได้ออกด้วยเครื่องกลั่นระเหยสารแบบหมุน (rotary evaporator) ศึกษาฤทธิ์ต้านอนุมูลอิสระของสารสกัดน้ำคั้นและสารสกัดเมทานอลของผลหนามแดงด้วยวิธี 1,1-diphenyl-2-picrylhydrazyl (DPPH) และ hydroxyl radical ($OH^{\bullet}$) scavenging assays โดยเปรียบเทียบกับสารมาตรฐานที่มีฤทธิ์ต้านอนุมูลอิสระ ได้แก่ แกลลิคแอซิด (gallic acid) และแมนนิทอล (mannitol) ตามลำดับ และศึกษาฤทธิ์ยับยั้งการเจริญของเซลล์ของสารสกัดหนามแดงด้วยวิธี 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay โดยทดสอบกับเซลล์มะเร็งเพาะเลี้ยง ได้แก่ เซลล์มะเร็งช่องปาก (KB), เซลล์มะเร็งเต้านม (MCF-7), เซลล์มะเร็งปากมดลูก (SiHa) และเซลล์มะเร็งตับ ($HepG_2$) และเซลล์ปกติไตลิง (Vero) ผลการศึกษาพบว่าสารสกัดน้ำคั้นและสารสกัดเมทานอลจากผลหนามแดงสุกมีฤทธิ์ในการต้านอนุมูลอิสระ แต่มีฤทธิ์ในการยับยั้งการเจริญของเซลล์มะเร็งเพาะเลี้ยงต่ำ ผลจากการศึกษานี้สรุปได้ว่าผลหนามแดงสุกมีคุณสมบัติต่อต้านอนุมูลอิสระ ซึ่งอาจนำไปใช้ประโยชน์ในการพัฒนาเป็นสารสำคัญเพื่อใช้ในการป้องกันโรคต่างๆ รวมทั้งโรคมะเร็งต่อไป *(วารสารโรคมะเร็ง 2562;39:6-15)*

คำสำคัญ: *Carissa carandas* Linn. หนามแดง ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ยับยั้งการเจริญของเซลล์ เซลล์มะเร็งเพาะเลี้ยง

Introduction

Many diseases are associated with oxidative stress caused by free radical. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism[1]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism[2]. Most of common ROS including superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) are inevitably produced as byproduct of normal aerobic metabolisms and play an important role in pathogenesis of several oxidative stress related diseases or disorder in human such as carcinogenesis, cardiovascular diseases, rheumatoid arthritis, ulcerative colitis, neurological degenerative diseases, atherosclerosis, ischemia, gastritis, aging, liver diseases, cancer and AIDS[1-4]. Antioxidants are vital substances which possess the ability to protect the human bodies from damage caused by free radical. The antioxidants can be effective in the prevention of the free radical formation by scavenging or promoting the decomposition and suppression of such diseases[1,3]. Cancer is an important public health problem that causes social and economic loss worldwide and also in Thailand. It was reported as a leading cause of death worldwide. Many researchers have

investigated the use of natural products for cancer prevention and treatments. Natural products used in traditional folk medicine have been the source of many medically beneficial drugs, as many medicinal plants have been shown to present interesting biological and pharmacological activities[6,7].

Carissa carandas Linn., belonging to the family Apocynaceae, is found to be widely distributed throughout Pakistan, India, Bangladesh, Srilunka, Java, Malaysia, Myanmar and Nepal. It is commonly known as "Nam Daeng" in Thai, Koromcha in Bengali and Karanda in English[8]. This plant is a climbing shrub, usually growing to 3-5 m high. Flowers are small, measuring 3-5 cm in diameter. The fruit is a berry, globose to broad ovoid in shape and contain many seeds. Most of its parts have medicinal importance. The fruit is very sour at maturity but it is sourish sweet when ripe. It can be eaten raw or stewed with sugar. In Thailand it is mainly used as pickles, however, it can also be made into jam, jelly, squash, syrup, chutney and puddings. Furthermore, the fruit is also used to make beverages, curries and tarts[9]. The fruits, leaves, barks and roots of *C. carandas* have been used for ethnomedicine in the treatment of human diseases, such as diarrhea, stomachic, anorexia, intermittent fever, mouth ulcer and sore throat, syphilitic pain, burning sensation, scabies and epilepsy[10]. Their prominent biological activities reported include antidiabetic, antimicrobial, cytotoxicity, anticonvulsant, hepatoprotective, antihyperlipidemic, cardiac depressant, analgesic, anti-inflammatory, antipyretic, antiviral properties and anticancer activity[8,11-13]. In this preliminary study, the juice and methanolic extracts of fresh ripe fruits of *C. carandas* were aimed to investigate for antioxidant and antiproliferative activities to support the possible use of *C. carandas* as chemopreventive and cancer therapeutic agents.

Materials and Methods

Plant materials

The ripe fruits of *C. carandas* were collected from Amphawa Market; Amphawa District, Samut Songkhram Province, Thailand. The fruits were washed with water and dried in the air at room temperature.

Preparation of crude extracts from *C. carandas* ripe fruits

Juice extract

Two kilograms of fresh ripe fruits of *C. carandas* were crushed in a juice extractor. The juice was collected and filtered through Whatman filter paper No.1. The combined filtrate was dried by lyophilization in a freeze dryer. The percentage of extract (% yield) was then calcu-

lated.

Methanolic extract

The residues of the crushed fruits were then extracted by maceration with methanol using electric shaker at room temperature for 7 days. The extract solution was filtered through Whatman filter paper No.1. The extractions were repeated for 2 times. The filtrates were combined and evaporated using a rotary evaporator at temperature of 40°C. The % yield was then calculated.

Determination of antioxidant activities

Antioxidant activities of the extracts of *C. carandas* fruit were measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl free radical scavenging assays.

DPPH free radical scavenging assay

The assay is on the basis of the measurement of the scavenging ability of antioxidant substances towards the stable radicals. The free radical scavenging assay with DPPH was performed as previously described by Pavithra K et al[2] with slight modification. The juice and methanolic extracts of *C. carandas* fruit were dissolved in methanol and diluted to various concentrations. One mL of 0.3 mM DPPH solution was then added to 0.5 mL of sample solution. The reaction mixture was shaken and left standing at room temperature in the dark for 30 minutes. Gallic acid was used as standard. The absorbance was measured using spectrophotometer at the wavelength of 517 nm. The percentage of radical scavenging activity of each extract was determined by comparison with the blank solution. The DPPH free radical scavenging activity of the extracts was measured in terms of percent inhibition and calculated by the following formula:

Percent (%) inhibition of DPPH radical = $[1-(A_{517}$ sample$/A_{517}$ control$)]$ x100

Where A_{517} sample is the absorbance in the presence of the extracts and A_{517} control is the absorbance in the presence of control.

Hydroxyl free radical scavenging assay

Hydroxyl radical scavenging assay is based on measurement of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid (TBA). Hydroxyl radical was generated from Fe^{3+}/ascorbate/EDTA/H_2O_2 system. This assay was performed as described by Hazra B et al[14] with a slight modification. Briefly, the reaction mixture contained 100 µL of 28 mM 2-deoxyribose, 200 µL of 1mM $FeCl_3$-1 mM EDTA, 100 µL of 10 mM H_2O_2, 100 µL of 1mM ascorbic acid, 500 µL of 20 mM KH_2PO_4-KOH buffer (pH 7.4) and 100 µL of various concentrations of the extracts were incubated at 37°C for 1 hour. The reaction mixture was added to 1.6 mL of 2.8% trichloroacetic acid (TCA) and

1.6 mL of 0.6% thiobarbituric acid (TBA). Subsequently, the mixture was heated in a boiling water bath for 15 minutes to develop the color. After cooling, the absorbance was measured at the wavelength of 532 nm. Mannitol, a classical OH scavenger, was used as a positive control. Percent inhibition was evaluated by comparing the extract and blank solution by the following formula:

Percent (%) inhibition of hydroxyl radical = [1-(A_{532} sample/A_{532} control)] x 100

Where A_{532} sample is the absorbance in the presence of the extracts and A_{532} control is the absorbance in the presence of control.

Determination of antiproliferative activity

Cell culture

The cancer cells used in the experiment were human epidermoid carcinoma cell (KB), human breast adenocarcinoma cell (MCF-7), human cervical carcinoma cell (SiHa) and human hepatocellular carcinoma cell ($HepG_2$) and non-tumorigenic cells (African green monkey kidney cell; Vero). All cells were cultured in Modified Eagle Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin and incubated at 37°C in a 5% CO_2 humidified incubator.

MTT colorimetric assay

The antiproliferative activity of *C. carandas* was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described by Mosmann T[15]. This assay is a quantitative colorimetric method to determine cell proliferation. It utilizes the yellow tetrazolium salt MTT which is metabolized by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple formozan reaction product. The cells were seeded in 96-well plate at a final density of $3x10^3$ cells/well in 100 μL of complete media. After incubating for 24 hours at 37°C in a 5% CO_2 humidified incubator, the cells were treated with 100 μL of various concentrations of the extracts. After incubating for 72 hours, 100 μL of MTT solution (5 mg/mL) was added to each well and the cells were incubated at 37°C for 4 hours. Then, the MTT mixture was removed and 100 μL of DMSO was added to each well to dissolve the formozan product. The absorbance was measured using a microplate reader at the wavelength of 570 nm. Effects of the extracts on cell proliferation were calculated using untreated cells as control. The 50% inhibition concentration (IC_{50} value) was determined by plotting the percentage of cell viability versus the extract concentrations.

Statistical analysis

All the assays were carried out in triplicates and the results were expressed as mean

values with standard deviation (SD).

Results

The fresh ripe fruits of *C. carandas* were extracted by crushing in the juice extractor. Then, the residues were extracted by maceration with methanol. The percentage yield of juice extract and methanolic extract were 4.54 and 3.26% w/w of fresh ripe fruits, respectively.

Antioxidant activities of the *C. carandas* extracts were measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl free radical scavenging assays. The DPPH radical scavenging activity of the juice and methanolic extracts of *C. carandas* at various concentrations of 25, 50, 100, 250, 500 and 1000 μg/mL showed a dose-dependent manner as shown in Figure 1. The juice and methanolic extracts of *C. carandas* exhibited slightly DPPH radical scavenging activity when compared with gallic acid (reference standard) as shown in Table 1. The IC_{50} values of gallic acid, the juice and methanolic extracts were 2.55 ± 0.08, 222.20 ± 7.76 and 242.82 ± 17.51 μg/mL, respectively.

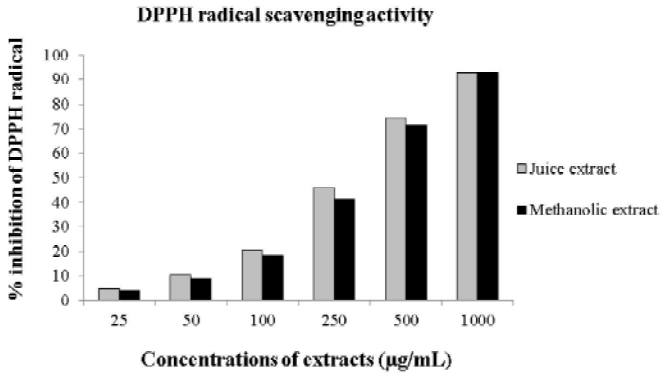

Figure 1 DPPH radical scavenging activity of the *C. carandas* extracts. The data show the percent inhibition of the extracts at various concentrations.

Table 1 DPPH radical scavenging activity of the extracts of *C. carandas* fruits

Samples	DPPH scavenging activity IC_{50} (μg/mL) Mean ± SD
Gallic acid	2.55 ± 0.08
Juice extract	222.20 ± 7.76
Methanolic extract	242.82 ± 17.51

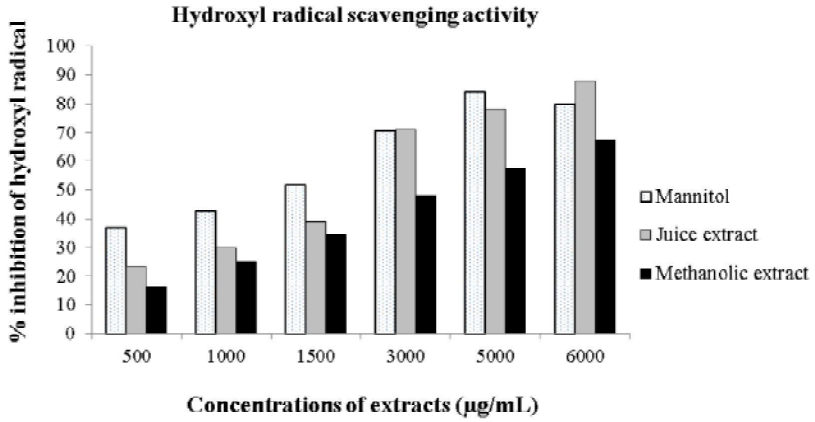

Figure 2 Hydroxyl radical scavenging activity of the *C. carandas* extracts. The data show the percent inhibition of the mannitol and the extracts at various concentrations.

Table 2 Hydroxyl radical scavenging activity of the extracts of *C. carandas* fruits

Samples	Hydroxyl scavenging activity IC_{50} (µg/mL) Mean ± SD
Mannitol	1188.64 ± 319.17
Juice extract	1812.63 ± 39.19
Methanolic extract	3112.90 ± 648.87

The hydroxyl radical scavenging activity of the juice and methanolic extracts of *C. carandas* at various concentrations of 500, 1000, 1500, 3000, 5000 and 6000 µg/mL showed a dose-dependent manner (Figure 2). The IC_{50} values of mannitol standard, juice extract and methanolic extract were 1188.64 ± 319.17, 1812.63 ± 39.19 and 3112.90 ± 648.87 µg/mL, respectively (Table 2). The result revealed that the juice extract exhibited hydroxyl radical scavenging activity when compared with mannitol (standard). Moreover, the finding indicated that the juice extract showed higher hydroxyl radical scavenging activity than the methanolic extract due to the lower IC_{50} value used to inhibit 50% activity of scavenging free radical.

Antiproliferative activity of the extracts of *C. carandas* fruit against KB, MCF-7, SiHa, $HepG_2$ and the non-tumorigenic Vero cells were investigated using the MTT colorimetric assay. As shown in Table 3, the juice extract showed the most antiproliferative activity against KB cells with IC_{50} value of 928.62 ± 80.89 µg/mL, followed by MCF-7, $HepG_2$ and SiHa with IC_{50} values of 1280.38 ± 59.04,

Table 3 Antiproliferative activity of the extracts of *C. carandas* fruit

Cells	Antiproliferative activity IC_{50} (μg/mL) Mean ± SD	
	Juice extract	**Methanolic extract**
KB	928.62 ± 80.89	74.21 ± 6.81
MCF-7	1280.38 ± 59.04	527.39 ± 87.68
$HepG_2$	2304.28 ± 81.91	472.26 ± 34.89
SiHa	3620.45 ± 91.69	595.42 ± 68.11
Vero	1509.49 ± 48.69	172.87 ± 8.01

2304.28 ± 81.91 and 3620.45 ± 91.69 μg/mL, respectively. In addition, the methanolic extract showed the most antiproliferative activity against KB cells with IC_{50} value of 74.21 ± 6.81 μg/mL, followed by $HepG_2$, MCF-7 and SiHa with the IC_{50} values of 472.26 ± 34.89, 527.39 ± 87.68 and 595.42 ± 68.11 μg/mL, respectively. These findings revealed that the methanolic extract showed higher antiproliferative activity than the juice extract.

Discussion and Conclusion

In this priliminary study, the antioxidant activities of *C. carandas* fruit extracts were evaluated using DPPH and hydroxyl radical (OH) scavenging assays. The results from the study indicated that the juice extract of *C. carandas* fruit showed slightly higher activities of DPPH and hydroxyl radical scavenging than the methanolic extract. The other researchers showed the similar findings[4,16-18]. A previous study reported that the major source of antioxidant capacity of *C. carandas* was both ascorbic acid and phenolic compounds[5].

However, the results from this study revealed that the juice and methanolic extracts of *C. carandas* fruits exhibited weak antioxidant activity compared with other studies. In this study, the IC_{50} values of the extracts in DPPH scavenging assay were 222.20 - 242.82 μg/mL but the other studies found that the IC_{50} values were 1.29 - 237.40 μg/mL[5,12,16]. It may be the different procedure of the extraction used. In this study, the fresh fruits of *C. carandas* were used for the extraction but dried fruits were used in the other studies[4-5,17-20].

In this study, the methanolic extract of

C. carandas fruit had higher antiproliferative activity against the four cancer cell lines than the juice extract. The previous study also found that the methanolic extract of *C. carandas* fruit showed antiproliferative activity against MCF-7 and $HepG_2$ cells[21]. However, in this study the juice and methanolic extracts of *C. carandas* fruit exhibited only weak antiproliferative activity. The American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of less than 30 μg/mL after the exposure time of 72 hours[22]. The findings of this study suggested that the fruits of *C. carandas* can be used as a source of natural antioxidant and may offer further application as chemopreventive agents.

Acknowledgement

The author would like to acknowledge the National Cancer Institute for the financial support in this research.

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