Middle-East Journal of Scientific Research 24 (8): 2418-2423, 2016
ISSN 1990-9233
© IDOSI Publications, 2016
DOI: 10.5829/idosi.mejsr.2016.24.08.10429

Quantitative Biochemical Analysis of Antioxidant Properties of *Carissa carandas* Fruit Ethanolic and *N*-hexane Extracts

[1]*I. Normaizatul Afizah,* [2]*W. Ahmad,* [2]*F. Jamshed and* [1]*S. Al-Jasabi*

[1]Faculty of Medicine, Insaniah University, Kedah, Malaysia
[2]Royal College of Medicine Perak, UniKl, Ipoh, Malaysia

Abstract: The present study has been conducted in the light of immense potential of medicinal plants used in various traditional systems. The aim was to determine quantitatively the antioxidant properties, total polyphenol contents, H_2O_2 reducing capacity, cytotoxic potentials, reducing power and DNA damage inhibition potential of an ethanolic and *n*-hexane extracts of *Carissa carandas* (*C. carandas*) fruits. Results showed significant antioxidant activities in both extracts compared to ascorbic acid, ASA and tert-butyl-1-hydroxytoluene (BHT) in 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging with IC_{50} of 1.44 µg/ml and 1.98 µg/ml of ethanolic extract and *n*-hexane extract respectively. H_2O_2 scavenging activities of both extracts, having IC50values higher than ASA or BHT. Total antioxidant activity and total phenolic content in fruit were also determined. Cytotoxic activities of the extractives were comparable to vincristine sulfate, having IC_{50} values of 3.43 and 2.66 of ethanolic extract and *n*-hexane extract respectively. Both extracts also completely protected pBR322 plasmid DNA from free radical-mediated oxidative stress in DNA damage inhibition assay. The antioxidant and DNA damage inhibition properties of C. carandas fruit extracts can be attributed to a high content of phenolic compounds (88.3 mg gallic acid equivalents/g dry weight of extract), estimated in the Folin–Ciocalteau assay. The high antioxidant and DNA damage inhibiting potential of C. carandas fruit could be used to develop antioxidant compounds for therapeutic applications.The present study provided data justifying the use of this plant for medicinal purposes.

Key words: *Carissa carandas* · Antioxidant Activity · Free Radical Scavenging · Polyphenols

INTRODUCTION

For decades, the utilization of herbal plants has drawn avalanche of interest as they could accommodate therapeutic response and are promising candidate to be developed as pharmaceutical product [1, 2].

Free radicals have been accused of initiating many serious diseases. These free radicals drive oxidative stress and transform the pathophysiological condition of the patient by acting on immune system [3, 4]. It has been known that phenolic and flavonoid compounds of the plant extracts are responsible for antioxidant and effects.

Herbal medicine is still the mainstay of maximum world population, mainly in the developing countries for primary health care not because they are inexpensive but also for better cultural acceptability, better compatibility with the human body and minimal side effects [5, 6]. The identification of active principles and their molecular targets from traditional medicine provides an enormous opportunity for drug development. The current study takes us a step ahead in the process of drug development as well as new validated treatment of a traditionally used medicinal plant. *C. carandas* a widely available medicinal plant belong to the family of Apocynaceae and prevalent in tropical and sub-tropical areas of the world.

Earlier studies [7-11] have shown that the extract of the plant possesses cardiotonic, antipyretic and antiviral activity. Various cardiac glycosides, a triterpenoidal constituent were reported from the root extract of this plant. Also, it was reported that the extract of the roots and leaves is effective remedies in the management and/or control of convulsions and epilepsy [12, 13]. However studies showed that root extract of *C. carandas* may produce its anticonvulsant effects via non-specific mechanisms.

Corresponding Author: S. Al-Jasabi, Faculty of Medicine, Insaniah University, Kedah, Malaysia.

It was reported also, that leaf and fruit of this plant has shown good anticancer activities [14, 15]. No studies have carried out to determine the antioxidant activity, total polyphenol contents, cytotoxic activity,reducing power and DNA damage inhibition of *C. carandas* fruit extracts. Therefore, this study was conducted to findout through quantitative biochemical analysis the antioxidant and other properties of the *C. carandas* fruit extract.

MATERIALS AND METHODS

Chemicals: All chemicals used in this study were of analytical grade and purchased from Sigma Co./USA.

Plant Materials: *Carissa carandas* ripe undamaged fruits were collected from Kuala Lumpur area, during April-July, cleaned by tap water 3 times then by phosphate buffer (pH.7.4), seeds were removed and the flesh of the fruits were dried using reflected dry air for 3 days and later dried in drier at 40°C for 7h (as recommended before [16]. The dried fruits were then ground into powder using high capacity grinding machine and stored in air-tight plastic container in dark and dry place for further investigation.

The dried powdered fruits (700 gm) of *C. carandas* was successively extracted in a Soxhlet extractor at 50°C-60°C temperature using 250 ml of ethanol followed by *n*-hexane. All extracts were filtered individually through filter paper and poured on petri dishes to evaporate the liquid solvents from the extract to get dry extracts which they were weighed and stored in air-tight container.

Free Radical Scavenging Activity: Free radical scavenging activities of extract and synthetic antioxidant substances used in the study prepared in ethanol and *n*-hexane at concentrations of 50, 100 and 200 µg/mL and determined in accordance with the method reported before [17], which is based on the principle of scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was added to the solutions prepared with plant extracts and standard antioxidant substances and stirred. Each mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm against a blank.

The total phenolic content of the plant extracts and the standard antioxidant materials was determined according to the Folin–Ciocalteu method [18]. Folin–Ciocalteu reagent was added to the extract and BHT solutions. After 5 min, Na_2CO_3 was added and the mixture was stored at room temperature for 2 h. The absorbance of the mixture was measured at 760 nm against water on a UV spectrophotometer. The results were calculated using the standard calibration curve of gallic acid ($R^2 = 0.9236$) and expressed as gallic acid equivalents (GAE mg/g).

$$\text{Absorbance } (\lambda 760) = 0.0026 \times [\text{Phenols } (\mu g)]$$

The total antioxidant capacity of the extracts was measured using commercial kits. In this method, the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid radical (ABTS) reacts with hydrogen peroxide and is oxidized to the $ABTS^+$ molecule. The ABTS radical loses its original blue and green colour. The intensity of the colour varies according to the quantity of antioxidants and their antioxidant capacity. The absorbance of this colour is measured spectrophotometrically at 660 nm [19].

The basis of this method depends on the oxidation of Fe with valence +2 to an iron complex with valence +3. Fe^{+3} forms a coloured complex with xylenol orange. Colour intensity varies according to the amount of oxidant in the sample. The absorbance of this colour is measured spectrophotometrically at 530 nm [20].

Hydrogen Peroxide Scavenging Capacity: The ability of the *C. carandus* fruit extracts to scavenge hydrogen peroxide was determined according to the method reported before [21]. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Extracts (100 µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of both *C. carandas* extracts and standard compounds were calculated as in the following:

$$\% \text{ Scavenged } [H_2O_2] = [(AC - AS)/AC] \times 100$$

The obtained extracts were filtered over Whattman No. 1 paper and the filtrate was collected, then solvent was removed by a rotary evaporator at 50°C [22].

Determination of Total Antioxidant Capacity: The total antioxidant capacity was evaluated by the phosphomolybdenum method [23]. 0.3 ml ofextract and sub-fraction in ethanol, ascorbic acid used as standard (5-200 µg/ml) and blank (ethanol) werecombined with 3 ml of reagent mixture separately and incubated at 95°C for 90 minutes. After cooling toroom temperature, the absorbance of each sample was measured at 695 nm

against the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by thefollowing equation:

$$A = (cX V) / m$$

where, A = total content of Antioxidant compounds, mg/gm plant extract, Equivalent c = the concentration of Ascorbic acid established from the calibration curve, mg/ml, V = thevolume of extract in ml,
m = the weight of crude plant extract, gm.

Total Antioxidant Activity-ferric Thiocyanate Method: The antioxidant activity of *C. carandas* fruit extracts and standards was determined according to the ferric thiocyanate method in linoleic acid emulsion [24].With this method peroxide formation occurred during the oxidation of linoleic acid oxidation. These compounds oxidized Fe_2+ to Fe_3+. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. The percent inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = [(AC- AS)/AC] \times 100$$

Total Phenolics Analysis: Total phenolic content of the fruit extract of *C. carandas* was measured employing the method described before [25] involving Folin-Ciocalteu reagent as oxidizing agent and gallic acidas standard [26].

Brine Shrimp Lethality Bioassay: Brine shrimp lethality bioassay [27, 28] technique was applied for the determination of general toxic properties of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a1-day *in vivo* assay. For the experiment, 4 mg of each of the ethanol and n-hexane soluble fractions were dissolved in DMSO and solutions of varying concentrations (100, 50, 25, 12.50, 6.25, 3.125, 1.563 µg/ml) were obtained by serial dilution technique using DMSO. Vincristine sulphate was used as positive control.

Determination of Reducing Property: The reducing power of the plant extracts was determined by a slightly modified method [29]. 1 ml of each extract concentration (0.1, 0.5 and 1 mg/ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K_3Fe (CN) 6] (2.5 ml, 1%). The mixtures were then incubated at 50 °C for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10%) were added to each mixture, which were then centrifuged for 10 min at1500 x g. The upper layer of the solutions (2.5 ml) were mixed separately with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance levels were measured at 700 nm using a spectrophotometer. Ethanol and *n*-hexane were used instead of the extracts as a controls. BHT (Butylated hydroxy toluene) and ASA were used as positive control and reducing power was reported as BHT and ASA equivalents per100 gm of dry sample.

DNA Damage Inhibition: Inhibition of DNA damage by ethanolic and *n*-hexane extracts of the fruits of *C. carandas* was tested by photolysing H_2O_2 with UV radiation in the presence of pBR322 plasmid DNA following the method reported before [30].

Statistical Analysis: Results were expressed as mean ± S.E.M and the intergroup variation was measured by one way analysis of variance (ANOVA) followed by Tukeys test. Statistical significance was considered at $P \leq 0.05$. The statistical analysis was done using Sigma state statistical software version 3.5.

RESULTS AND DISCUSSION

In this study, the antioxidant activity of *C. carandas* fruit extracts were compared to butylated hydroxy anisole (BHA) and ascorbic acid (ASA). The antioxidant activity of the extracts, ascorbic acid and BHA was also evaluated in a series of the following *in vitro* tests i.e. H_2O_2 radicals scavenging and total antioxidant activity (ferric thiocyanate method) [31].

All the extractives of *C. carandas* fruit were subjected to free radical scavenging activity using DPPH and also, using ASA and (BHT) as reference standards. In this investigation, the ethanol and *n*-hexane extracts showed significant free radical scavenging activitywith IC_{50} value of 1.75 µg/ml and 2.38 µg/ml respectively.

The scavenging ability of ethanol and *n*-hexane extracts of *C. carandas* on hydrogen peroxide are compared with BHA and ASA as standards. The *C. carandas* fruit extracts were capable of scavenging hydrogen peroxide in an amount dependent manner. 100 µg of ethanol and *n*-hexane extracts of *C. carandas* fruit exhibited17.6% a scavenging activity of ethanolic extract and 22.7% for *n*-hexane, on hydrogen peroxide. On the other hand, using the same amounts, BHA and ASA exhibited 47.22% and 43.55% hydrogen peroxide

scavenging activity. Results show that the scavenging activity values on hydrogen peroxide of 100 µg of the extracts of *C. carandas* fruits less than that of BHA and ASA.

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells [32, 33]. Thus, the removing of H_2O_2 is very important for antioxidant defence in cell or food systems.

All the extractives of *C. carandas* fruit were subjected to free radical scavenging activity using DPPH by using (ASA) and BHA as reference standards. In this investigation, both ethanoland *n*-hexane extracts showed significant free radical scavenging activity with IC_{50} value of 4.66 µg/ml, while for *n*-hexane it was 8.42 µg/ml.

Total antioxidant capacity of the different extracts of *C. carandas* fruitwas evaluated by the phosphomolybdenum method [34] and was expressed as ascorbic acid equivalents (AAE) per gram of fruitextract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid:

$(y = 0.002x + 0.001; R2 = 0.997)$.

n-hexane extract of *C. carandas* fruit was found to possess the higher total antioxidant capacity.

All the extractives of *C. carandas* fruit were tested for total phenolic contents. The higher phenolic content was found in *n*-hexane, 18.22± 2.7 mg/gm, while it was (11.71±1.80 mg/gm in ethanolic extract of GAE/gm of extractives).

In the brine shrimp lethality bioassay, the LC_{50} values of ethanol and *n*-hexane extracts of *C. carandas* fruits were found to be 2.818 and 1.995 respectively.

The fruit extracts also showed some level of cytotoxic activities on *Artemia salina.* Hence, it could be concluded that the ethanolic and *n*-hexane extracts of fruits of *C. carandas* has antioxidant as well as cytotoxic activities.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe3+/ferricyanide complex used in this method to ferrous form. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of ferrous ions. Increased absorbance of the reaction mixture indicated increased reducing power of the extracts.

The reducing power of both extracts increased with the increase in their concentrations. At 1 mg/ml concentration of the standard BHT the absorbance obtained was 1.97. At the same concentration *C. carandas* ethanolic extract was found to have the absorbance value 2.83 while *n*-hexane extract has 2.21. Thus, the extracts of *C. carandas* showed that they possessed very high antioxidant properties.

The study had proved that the presence of these phytochemicals enhances the efficacy and dilutes toxicity also. The total phenolic content of selected splant species was done and established that antioxidant activity was closely correlated with phenolic content [35]. During the present work, it was found that *C. carandas* exhibited higher antioxidant activity with higher phenolic content. So these findings are in agreement with previous reports that there is linear relation between antioxidant activity and total phenolic contents.

Table 1: I C_{50} values of *C. carandas* ethanolic and *n*-hexane extract and the standards of DPPH free radicals

Name of Standard and Sample	*IC_{50} (µg/ml)*
Ascorbic acid (ASA)	0.672
Butylated Hydroxy Toluene (BHT)	0.722
Ethanol	1.477
n-hexane	1.998

REFERENCES

1. Aderogba M., E. Okoh and T. Idowu, 2005. Evaluation of antioxidant activity of the secondary metabolites from Poliostigma reticulatum (DC) hochst. Journal of Biological Sciences, 5: 239-242.
2. Rabaud, C., H. Tronel, S. Fremont, T. May, P. Canton and J. Nicolas, 1997. Free radicals and HIV infection. Annales de Biologie Clinique, 55: 565-571.
3. Malorni, W., R. Rivabene, B. Lucia, R. Ferrara, A. Mazzone and R. Cauda, 1998. The role of oxidative imbalance in progression to AIDS Effect of the thiol supplier N-acetylcysteine. AIDS Research and Human Retroviruses, 14(17): 1589-1596.
4. Robert, A. and B. Meunier, 1998. Is Alkylation the Main Mechanism of Action of the Antimalarial Drug Artemisinin? Chemical Society Review Articles, 27(4): 273-274.
5. Pauli, G., R. Case, T. Inui, Y. Wang, S. Cho and H. Fischer, 2005. New perspectives on natural products in TB drug research. Life Sciences, 78(5): 485-494.

6. Shah, P., 2005. The need for new therapeutic agents: What is in the pipeline? Clinical Microbiology and Infection, 11(3): 36-42.
7. Rios, J. and M. Recio, 2005. Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology, 100(1&2): 80-84.
8. Da-Silva, J., M. De-Souza, S. Matta, M. De-Andrade and F. Vidal, 2006. Correlation analysis between phenolic levels of brazilian propolis extracts and their antimicrobial and antioxidant activities. Food Chemistry, 99: 431-435.
9. Majhenic, L., M. Skerget and Z. Knez, 2007. Antioxidant and antimicrobial activity of guarana seed extracts. Food Chemistry, 104(3): 1258-1268.
10. Pereira, J., I. Oliveira, A. Sousa, P. Valentao and P. Andrade, 2007. Walnut (*Juglans regia* L.) leaves: Phenolic com-pounds, antibacterial activity and antioxidant potential of different cultivars. Food and Chemical Toxicology, 45: 2287-2295.
11. Morton, J., "Fruits of Warm Climates". 1987, Miami, FL: Julia F. Morton.
12. Hegde, K., S. Thakker, A. Joshi, C. Shastry and K. Chandrashekhar, 2009. Anticonvulsant activity of *Carissa carandas* Linn. root extract in experimental mice. Tropical Journal of Pharmaceutical Research, 8(2): 117-125.
13. Dhawan, B. and G. Patnaik, 1985. Investigations on some new cardio-active glycosides. Indian Drugs, 22(6): 285-290.
14. Rajasekaran, A., V. Jeyasudha, B. Kalpana and B. Jayakar, 1999. Preliminary phytochemical and antipyretic evaluation of *Carissa carandas*. Indian J. Nat Prod., 15(1): 27-29.
15. Taylor, R., J. Hudson, N. Manandhar and G. Tower, 1996. Antiviral activities of medicinal plant of Southern Nepal. J Ethnopharmacol, 53(2): 97-104.
16. Rastogi, R., M. Vohra, R. Rastogi and M. Dhar, 1966. Studies on *Carissa carandas* Linn. Part I. Isolation of the cardiac active principles. Indian J Chem, 4: 132.
17. Kirtikar, K. and B. Basu, 2003. Indian medicinal plant. Volume II, Lalit Mohan Basu, Allahabad, pp: 1546.
18. Begum, S., S. Syed, B. Siddiqui, S. Sattar and M. Choudhary, 2013. Carandinol: First isohopane triterpene from the leaves of *Carissa carandas* L. and its cytotoxicity against cancer cell lines. Phytochem Lett, 6(1): 91-95.
19. Sharma, A., R. Tiwari, A. Kaushik, L. Tyagi, K. Shankar and T. Virmani, 2007. Standardisation of *Carissa carandas* Linn: A drug used in Indian system of medicine as per WHO Guidelines. Cont. J. Pharm. Sci., 1: 9-14.
20. Devmurari, V., P. Shivanand, M. Goyani, S. Vaghan and N. Jivani, 2009. A review: Carissa congesta: Phytochemical constituents, traditional use and pharmacological properties. Pharmacogn Rev., 3(6): 375.
21. Pino, J., R. Marbo and C. Vázquez, 2004. Volatile flavor constituents of Karanda (*Carissa carandas* L.) fruit. J Essent Oil Res, 16(5): 432-4.
22. Gaurav, S., N. Navneet, R. Sandeep, P. Singh, P. Amit and N. Manisha, 2010. Effect of aqueous leaves extract of *Carissa carandas* linn. on blood glucose levels of normoglycemic & alloxan induced diabetic wister rats. Int. J. Curr. Pharm. Res., 2(3): 65-7.
23. Itankar, P., S. Lokhande, P. Verma, S. Arora, R. Sahu and A. Patil, 2011. Antidiabetic potential of unripe *Carissa carandas* Linn. fruit extract. J Ethnopharmacol., 135(2): 430-3.
24. Bhaskar, V. and N. Balakrishnan, 2009. Analgesic, anti-inflammatory and antipyretic activities of Pergularia daemia and *Carissa carandas*. Daru. J. Pharm Sci., 17(3): 168-74.
25. Hati, M., B. Jena, S. Kar and A. Nayak, 2014. Evaluation of anti-inflammatory and anti-pyretic activity of *Carissa carandas* L. leaf extract in rats. J Pharm Chem Biol. Sci., 1(1): 18-25.
26. Anupama, N., G. Madhumitha and K. Rajesh, 2014. Role of dried fruits of *Carissa carandas* as anti-inflammatory agents and the analysis of phytochemical constituents by GC-MS. Biomed Res Int., 2014: 512369.
27. Bhaskar, V. and N. Balakrishnan, 2009. Hepatoprotective activity of laticiferous plant species (Pergularia daemia and *Carissa carandas*) from Western Ghats, Tamilnadu, India. Pharm Lett, 1(2): 130-42.
28. Saha, R., L. Hossain, U. Bose and A. Rahman, 2010. Europharmacological and diuretic activities of Carissa carandas linn. Leaf. Pharmacologyonline, 2: 320-7.
29. Middlemiss, D. and S. Watson, 1994. A medicinal chemistry case study: An account of an angiotensin II antagonist drug discovery programme. Tetrahedron, 50: 13049-80.
30. Shamim, S. and S. Ahmad, 2012. Pharmacodynamic study on acute hypotensive activities of *Carissa carandas* extract in normal rats. Pak J. Pharm. Sci., 25(3): 577-82.
31. Islam, M., S. Rahman, M. Ahmed, P. Das, M. Tabibul and M. Islam, 2012. Antinociceptive activity studies with methanol extract of *Annona reticulata*

L. (Annonaceae) and *Carissa carandas* L. (Apocynaceae) leaves in Swiss albino mice. Adv Nat. Appl. Sci., 6(8): 1313-8.

32. Halliwell, B. and J. Gutteridge, 1999. Free radicals in biology and medicine. 3rd ed. 1999, Oxford: Oxford University Press.
33. Mau, J.L., C.N. Chang, S.J. Huang and C.C. Chen, 2004. Antioxidant properties of methanolic extracts from Grifola frondosa, Morchella esculanta and Termitomyces albuminosus mycelia. Food Chem, 87:111-8.
34. Cakir, A., M.A.A. Yildirim, M.E. Duru, M. Harmandar and C. Kazaz, 2003. Isolation and Characterization of antioxidant Phenolic Compounds from the Aerial Parts of Hypericum hyssopifolium L. By activity-guided fractionation. J. Ethnophar, 87: 73-83.
35. Mehmood, M., N. Anila, S. Begum, S. Syed, B. Siddiqui and A. Gilani, 2014. Pharmacological basis for the medicinal use of *Carissa carandas* in constipation and diarrhea. J. Ethnopharmacol., 153(2): 359-67.