Asian Journal of Chemistry; Vol. 25, No. 7 (2013), 3913-3916

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13842

Comparative Study of Antioxidant, Antimicrobial and α-Amylase Inhibitory Activities of Leaves, Fruits and Seeds of *Carissa opaca*

DILDAR AHMED* and MUHAMMAD HAMMAD

Department of Chemistry, Forman Christian College (A Chartered University), Lahore, Pakistan

*Corresponding author: E-mail: dildarahmed@gmail.com

(*Received*: 4 April 2012; *Accepted*: 18 January 2013) AJC-12722

Carissa opaca Stapf ex Haines is a medicinal plant found in different hilly areas of Indo-Pakistan subcontinent. The present study aimed to compare antioxidant, antimicrobial and α-amylase inhibitory activities of leaves, fruits and seeds of *C. opaca*. The free radical scavenging activities of leaves, fruits and seeds were 89.84, 9.44 and 3.85 %. The leaves also showed much higher antioxidant activity determined by phosphomolybdate assay than fruits and seeds. All the three parts of the plant showed considerable α-amylase inhibitory activities but the leaves proved to be much stronger than fruits and seeds; their IC_{50} values being 5.03, 79.92 and 80.07 mg/mL respectively; while that of acarbose standard it was 1.20 mg/mL. Antimicrobial activities of leaves, fruits and seeds were investigated against *Salmonella typhi, Escherichia coli, Bacillus subtilis, Shigella sonnei, Staphylococcus aureus* and *Pseudomonas aeruginosa*. The antimicrobial activities of leaves against different microorganisms were in general better than those of fruits and seeds. However, fruits showed remarkable efficacy against *S. typhi* which was almost equal to antibiotic cefixime and clarithromycin.

Key Words: *Carissa opaca*, α-Amylase inhibitory, Antioxidant, Antimicrobial.

INTRODUCTION

Carissa opaca Stapf ex Haines (family apocynaceae) is a shrub found in many hilly areas of Indo-Pakistan subcontinent[1-6]. The plant has found a number of folkloric applications. In ethnomedicine, *Carissa opaca* is used against a number of diseases and conditions. Its fruit is considered to be aphrodisiac[7,8]. The leaves are used for the treatment of liver disorders such as jaundice and hepatitis. A decoction prepared by boiling its leaves with roots of *Segeretia brandrethiana* is taken two times a day[9]. They are also used to cure asthma, known as stimulant and cardio-tonic and is used in tanning[10-12]. The paste made of the roots of *Carissa opaca* is applied on wounds and injuries for fast healing. The roots are also considered to be purgative[13]. The extracts of other species of the genus, *C. carandas* and *C. edulis*, have been found to possess good anti-diabetic activities[14,15]. Fruits and leaves of *C. opaca* have been shown by different workers to possess considerable antioxidant activities[16,17]. The ethanolic extracts of the leaves and fruits of the plant were found to have notable antimicrobial activities against a number of Gram-positive and Gram-negative bacteria[18]. The plant has also been shown to possess flavonoids, tannins, alkaloids, phlobatannins, terpenoids, coumarins, anthraquinones, cardiac glycosides, isoquercetin, hyperoside, vitexin, myricetin and kaempherol[19]. A sesquiterpenoid called carrisone ($C_{15}H_{24}O_2$) has been isolated from *Carissa opaca* the structure of which has been confirmed by X-ray diffraction and NMR spectroscopy[20]. The GC-MS analysis of the oil from flowers of *C. opaca* has shown the presence of benzyl salicylate (6.0 %), benzyl benzoate (4.6 %) and (E,E)-α-farnesene (3.5 %) in it[21]. Hydro-distillation of the roots yielded 2-hydroxyacetophenone as a major component (89.5 %)[22].

In continuation of our work on *Carissa opaca*[18], the present research aimed to investigate antioxidant, antimicrobial and α-amylase inhibitory activities of the leaves, fruits and seeds of the plant.

EXPERIMENTAL

The leaves and fruits of the wild shrub *Carissa opaca* were collected from a village of Abbottabad (Hazara), Pakistan, in May 2009 and identified by Dr. Syed Muqarrab Shah, the taxonomist of Hazara University, Mansehra. The leaves and fruits were dried in shade at room temperature for 15 days. Seeds were obtained by carefully removing the fruit pulp. The dried leaves, fruit pulp and seeds were ground to get finely divided powders which were extracted in methanol for two weeks at room temperature. The extracts were filtered and the solvent was evaporated on rotary evaporator (Buchi A-210) to obtain dried methanolic extracts.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical and ascorbic acid were purchased from MP biomedicals, (France) and α-amylase (Aspergillus *oryzae*) from Unichem and acarbose from Baeyer, Pakistan. All other substances were of analytical grades. UV-Visible Spectrophotometer UVD-3200 Labomed, Inc. was used to determine absorbance.

α-Amylase inhibitory assay: The α-amylase inhibitory activities of the methanolic extracts of the leaves, fruits and seeds of *C. opaca* were determined as per a reported method[23]. In the assay, 0.5 % aqueous potato starch solution was used as a substrate. The samples of leaves, fruits and seeds were prepared in dimethyl sulfoxide in a range of concentrations (1.0 to 150 mg/mL). The α-amylase solution was prepared by dissolving 1 mg of the enzyme in 100 mL of 20 mM phosphate buffer (pH 6.9). The 3,5-dinitrosalicylic acid (DNS) solution was used as the colouring reagent of reaction. In a test tube, 0.5 mL of a sample was mixed with 0.5 mL of the enzyme solution and after incubating the mixture for 30 min at 25 ºC, 1 mL of starch solution was mixed and the mixture was allowed to incubate for 3 min at 25 ºC. Then, after adding 1 mL of the DNS (3,5-dinitrosalicylic acid) solution (20 mL of 96 mM 3,5-dinitrosalicylic acid, 12 g of sodium potassium tartrate in 8 mL of 2 M NaOH and 12 mL of water) solution, the tube was covered and heated in a water bath for 15 min at 85 ºC. The mixture was then diluted with 9 mL water. The absorbance was measured at 540 nm on a UV-Visible spectrophotometer. For control, plant sample was replaced by equal volume of DMSO and for blank, the starch solution was added after the addition of DNS solution. Remaining procedures were same. To compare the alpha-amylase inhibitory activities of the sample, acarbose was employed as a positive control. The percentage inhibition was calculated by the formula:

$$\% \text{ Inhibition} = [(A_c - A_s)/A_c] \times 100$$

where, A_c and A_s are the absorbances of the control and sample respectively.

Microorganisms: In the present study, *Salmonella typhi, Escherichia coli, Bacillus subtilis, Shigella sonnei, Staphylococcus aureus, Pseudomonas aeruginosa* were used to compare antimicrobial activities of the leaves, fruits and seeds extracts of *C. opaca* with standard antibiotic drugs, cefixime, levofloxacin and clarithromycin.

Agar well diffusion assay: The antimicrobial activities of the methanolic extracts of leaves, fruits and seeds were determined by agar well diffusion method as recommended by clinical and laboratory standards institute (CLSI)[24] and described as earlier[25]. The bacterial suspensions were prepared by adding 4-5 colonies of bacteria into 5 mL autoclaved normal saline (NS) solution and comparing its turbidity with 0.5 % McFarland solution. The microorganisms were inoculated on the Petri plates (90 mm diameter) containing Mueller-Hinton Agar (MHA) with the help of sterile cotton swab following the recommended usual procedure[25,26]. Wells were then made in the inoculated MHA plates with the help of cork borer (9 mm) and labeled. Then, 100 µL of leaves, fruits and seeds extracts (0.1 g/100 mL each), 6 % phenol and 0.1 g/mL of each of cefixime, clarithromycin and levofloxacin (positive controls) and 95 % methanol (negative control) were transferred into their respective wells. The plates were incubated for 24 h at 37 ºC; subsequently zones of inhibition were measured using Vernier calipers[18,25]. All experiments were repeated thrice and mean were calculated.

Free radical scavenging activities by DPPH assay: Free radical scavenging activities of the methanolic extracts of leaves, fruits and seeds were determined as per the reported method[27,28]. The stock solution of DPPH radical was prepared by dissolving its 24 mg in 100 mL of methanol and stored at 20 ºC for furthermore use. The working solution was prepared by diluting DPPH solution with methanol to obtain an absorbance of 0.980 ± 0.02 at 517 nm. The sample solutions (2 mg/mL) were prepared by dissolving 20 mg of each of the leaves, fruits and seeds extracts in 10 of methanol. The positive control was prepared by dissolving 20 mg of ascorbic acid in the 10 mL of methanol. The negative control consisted of 3 mL of DPPH working solution and 100 µL of methanol. The DPPH working solution (3 mL) was mixed with 100 µL of the samples with varying concentrations (25-250 µg/mL) in glass vials. After incubating the vials for 30 min in the dark, absorbance was measured at 517 nm on a UV-Visible spectrophotometer. The free radical scavenging activity of each sample was calculated by using the formula:

$$\% \text{ Scavenging activity} = [(A_0 - A_s)/A_0] \times 100$$

where A_0 and A_s are absorbances of negative control and sample respectively.

Total antioxidant activities by phosphomolybdate assay: Total antioxidant activities of the methanolic extracts of leaves, fruits and seeds of *C. opaca* were determined as per the reported method[28,29]. For this assay, 0.1 mL of each sample solution was mixed with 1 mL of the phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the test tubes were capped with silver foil and incubated in water bath at 95 ºC for 90 min. Then, after cooling the mixture to room temperature, its absorbance was measured at 765 nm against a blank. Ascorbic acid was used as a standard and the results were expressed as µg of ascorbic acid equivalent per mg of the methanolic extract.

Statistical analysis: To ensure reproducibility, all the experiments were performed at least thrice and statistical mean was calculated. One way ANOVA was also applied and IC_{50} and EC_{50} values were calculated using the standard methods.

RESULTS AND DISCUSSION

α-Amylase inhibitory activities: The α-amylase inhibitory activities of the methanolic extracts of leaves, fruits and seeds of *Carissa opaca* were determined as per the method proposed by Bernfeld[30]. The results are shown in Table-1 and Fig. 1. Acarbose was used as a standard to have an estimation of the efficacy of the plant extracts. In the assay the substrate (starch) is attacked by the enzyme to produce maltose which reacts with DNS to form a coloured product which allows monitoring of the reaction spectrophotometrically. The methanolic extracts of leaves, fruits and seeds of the plant exhibited pretty good α-amylase inhibitory activities. However, the leaves proved to be most effective (IC_{50} 5.03 mg/mL). Their efficacy was close to that of acarbose (1.2 mg/mL). The extracts exhibited anti-enzymatic activity in a dose dependent manner (Fig. 1).

TABLE-1
α-AMYLASE INHIBITORY ACTIVITIES AND IC_{50} (mg/mL) VALUES OF THE METHANOLIC EXTRACTS OF LEAVES, SEEDS AND FRUITS OF *Carissa opaca.*

S. No	Leaves		Seeds		Fruits		Acarbose	
	mg/mL	Inhibition (%)	mg/mL	Inhibition (%)	mg/mL	Inhibition (%)	mg/mL	Inhibition (%)
1.	1	07.45 ± 1.29	2	02.23 ± 0.83	10	03.19 ± 1.45	0.10	03.53 ± 1.65
2.	2	21.36 ± 1.98	4	06.46 ± 2.76	20	09.76 ± 3.68	0.20	14.11 ± 0.48
3.	3	33.90 ± 1.67	6	11.88 ± 2.90	30	17.45 ± 2.99	0.40	20.63 ± 0.74
4.	4	40.54 ± 2.88	8	14.12 ± 3.96	40	25.50 ± 2.75	0.60	29.72 ± 1.23
5.	5	51.33 ± 3.10	10	17.34 ± 4.01	50	30.98 ± 2.15	0.80	37.44 ± 2.22
6.	6	59.96 ± 0.17	20	25.56 ± 3.37	60	35.04 ± 2.69	1.18	45.51 ± 0.49
7.	7	67.70 ± 1.43	40	33.54 ± 3.15	70	41.11 ± 0.56	1.47	54.89 ± 0.28
8.	8	75.21 ± 1.65	60	42.07 ± 2.79	80	47.22 ± 1.88	1.84	66.77 ± 0.45
9.	9	82.78 ± 2.21	80	51.31 ± 4.27	90	52.66 ± 1.49	2.30	78.00 ± 0.36
10.	10	98.01 ± 1.32	100	64.98 ± 0.71	100	60.89 ± 2.79	2.80	86.95 ± 0.92
11.	-	-	110	73.46 ± 1.98	110	69.33 ± 3.12	3.60	93.18 ± 0.86
12.	-	-	120	81.23 ± 1.47	120	77.98 ± 0.63	-	-
13.	-	-	130	93.19 ± 2.48	130	83.85 ± 0.77	-	-
14.	-	-	-	-	140	89.12 ± 4.50	-	-
15.	-	-	-	-	150	95.54 ± 3.23	-	-
IC_{50}	5.03		79.92		80.07		1.20	

TABLE-2
COMPARISON OF ANTIMICROBIAL ACTIVITIES OF THE METHANOLIC EXTRACT OF LEAVES, FRUITS AND SEEDS OF *Carissa opaca* AND KNOWN ANTIBIOTICS AGAINST MICROORGANISMS (MEAN ZONES OF INHIBITION in mm)*^

	Leaves	Fruits	Seeds	Phenol 6 %	Cefixime	Levofloxacin	Clarithromycin
Salmonella typhi	17.3	20.6	10.2	29.00	21.5	34.00	22.10
Escherichia coli	11.5	11.1	12.0	18.30	-	-	-
Bacillus subtilis	16.5	14.4	9.5	25.20	32.0	35.25	23.15
Shigella sonnei	14.0	10.1	6.3	21.00	-	-	-
Staphylococcus aureus	14.2	11.1	12.00	-	35.0	39.10	21.50
Pseudomonas aeruginosa	14.0	10.0	6.30	-	29.0	37.75	26.00

*Concentrations of the methanolic extracts were ^Mean zones of Inhibition values are mean of three experiments

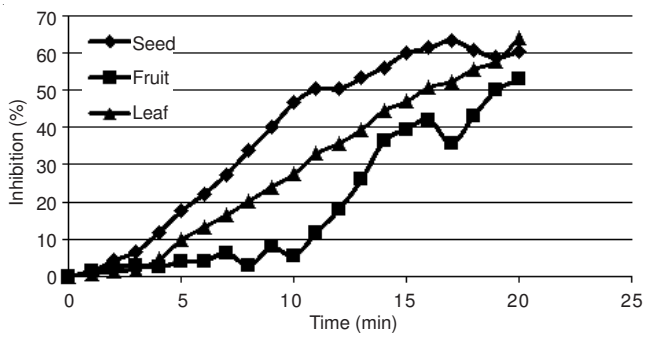

Fig. 1. Change in α-amylase inhibitory effect of leaves, fruits and seeds of *Carissa opaca* with time

Antimicrobial activities: The antimicrobial activities of the methanolic extracts of the leaves, fruits and seeds of *Carissa opaca* against a number of microorganisms were determined along with three antibiotics cefixime, levofloxacin and clarithromycin, using agar well diffusion assay and the results are displayed in Table-2. The microorganisms used included *Salmonella typhi*, *Escherichia coli, Bacillus subtilis, Shigella sonnei, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Table-2 exhibits that the leaves, in general, showed better potency than fruits and seeds. The fruits, however, showed highest activity (20.6 mm) against *Salmonella typhi* as compared to leaves and seeds. Notably, this is the highest activity that a sample showed against any of the tested microbes. The activity of the fruits against *S. typhi* is almost equal to that of cefixime (21.5 mm) and clarithromycin (22.1 mm). Cefixime is a third generation cephalosporin and is effective in typhoid[31]. Consumption of the fruits of *C. opaca*, therefore, can be beneficial for typhoid patients. The leaves also showed notable efficacy against *S. typhi*. In addition, both leaves and fruits extracts showed good activity against *B. subtilis*. The seed extract, in general, was least effective, while leaves have also exhibited considerable toxicity against *S. sonnei, S. aureus* and *P. aeruginosa*. A decoction of leaves should, therefore, be useful in the treatment of conditions caused by these pathogens.

Free radical scavenging activities by DPPH assay: The free radical scavenging activities of methanolic extracts of leaves, fruits and seeds of *C. opaca* were studied by DPPH assay and the results are shown in the Table-3. The DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical ($DPPH^{\bullet}$) which shows maximum absorbance at 517 nm. It accepts a hydrogen radical or an electron to form a diamagnetic molecule and the purple colour of the radical changes into pale yellow [27,32]. Table-3 shows that the leaves exhibited very high free radical scavenging activity (89.84 %) than both fruits (9.44 %) and seeds (3.85 %) which was comparable to that of ascorbic acid (95.22 %). The EC_{50} value of the leaves was 499.95 µg/mL which was however much less than that of ascorbic acid (99.91 µg/mL). There was good correlation between the free radical scavenging activity determined by DPPH assay and total antioxidant activity determined by phosphomolybdate assay.

TABLE-3
% FREE RADICAL SCAVENGING POTENTIAL OF LEAVES, FRUITS AND SEEDS OF *Carissa opaca* ALONG WITH ASCORBIC ACID STANDARD DETERMINED BY DPPH METHOD

Sample	Scavenging activity (%)	EC_{50} (µg/mL)
Leaves	89.84	499.95
Fruits	09.44	-
Seeds	03.85	-
Ascorbic acid	95.22	99.91

Total antioxidant activities by phosphomolybdate assay: The phosphomolybdate assay is based on reduction of molybdenum(VI) to molybdenum(V) by the action an antioxidant. The formation of the green phosphomolybdate(V) complex is followed spectrophotometrically[29]. The results of the present study are shown in Fig. 2. The methanolic extract of the leaves exhibited remarkable antioxidant activity which is much higher than those of fruits and seeds which indicate the presence of good antioxidant substance in leaves of this plant and should explain in part the ethnomedicinal uses of the leaves.

Fig. 2. Antioxidant potential of methanolic extract of leaves, fruits and seeds of *Carissa opaca* determined by phosphomolybdate assay in teerms of ascorbic acid equivalents

Conclusion

The methanolic extracts of leaves, fruits and seeds of *Carissa opaca* have pretty good α-amylase inhibitory activities which show presence of antienzymatic natural products in them. Remarkable high efficacy of the fruits against *S. typhi* may indicate the presence of such compounds in fruits which are effective against this pathogen. The present study highlights the need of bioassay guided isolation of bioactive natural products from this medicinal plant which may prove to be good candidates for the future drug discovery.

REFERENCES

1. R.D. Gaur, Flora of the District Garhwal North West Himalaya. Transindia: Srinagar, Uttaranchal (1999).
2. J.D. Hooker, Flora of British India, 3. Reeve and Co: Ashford, Kent, (1882).
3. S.K. Rai, G.R. Mallavarapu, S. Pandey-Rai, S. Srivastava, D. Singh, R. Mishra and S. Kumar, *Flav. Fragr. J.*, **21**, 304 (2006).
4. S. Nazimuddin and M. Qaiser, in eds.: E. Nasir and S.I. Ali, Apocynaceae, Flora of Pakistan Karachi: Department of Botany, University of Karachi, pp. 11-13 (1983). (http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250077072)
5. S. Handoo, A Survey of Plants Used for Wound Healings in Animals, Vet Scan (Online Veterinary Journal), vol. 1, No 1, Article 2, (2006). (http://www.kashvet.org/vetscan, ISSN 0973-6980).
6. D. Joshi, Plants of Kumaon, http://www.uttaranchal.org.uk/articles.php, (2010).
7. S.S. Ahmad, F. Mahmood, Z. Dogar, Z.I. Khan, K. Ahmad, M. Sher, I. Mustafa and E.E. Valeem, *Pak. J. Bot.*, **41**, 2105 (2009).
8. D. Acharya and M.K. Rai, Traditional Knowledge for Curing Various Ailments Among Gonds and Bharias of Patalkot Valley, M.P., India, (2009).
9. A.M. Abbasi, M.A. Khan, M. Ahmad, M. Zafar, H. Khan, N. Muhammad and S. Sultana, *Afr. J. Biotechnol.*, **8**, 1645 (2009).
10. A. Jabeen, M.A. Khan, M. Ahmad, M. Zafar and F. Ahmad, *Afr. J. Biotechnol.*, **8**, 777 (2009).
11. C. Parmar and M.K. Kaushal, Wild Fruits. Kalyani: New Delhi, (1982).
12. T. Ravishankar, L. Vedavalli, A.A. Nambi and V. Selvam, Role of Tribal People in the Conservation and Utilization of Plant Genetic Resources. MS Swaminathan Research Foundation: Chennai (1991).
13. K. Dinesh, H.C. Ripathi, S.K. Tandon, J. Lal and J.K. Malik, *Indian J. Vet. Surgery*, **18**, 65 (1997).
14. P.R. Itankar, S.J. Lokhande, P.R. Verma, S.K. Arora, R.A. Sahu and A.T. Patil, *J. Ethnopharmacol.*, **135**, 430 (2011).
15. F.K. El-Fiky, M.A. Abou-Karam and E.A. Afify, *J. Ethnopharmacol*., **50**, 43 (1996).
16. S. Sahreen, M.R. Khan and R.A. Khan, *Food Chem.*, **122**, 1205 (2010).
17. M.A. Khan, D. Shahwar, N. Ahmad, Zaheer-ud-din Khan and M. Ajaib, *Asian J. Chem.*, **22**, 379 (2010).
18. D. Ahmed, A. Waheed, M.A. Chaudhary, S.R. Khan, A. Hannan and M. Barkaat, *Asian J. Chem.*, **23**, 2072 (2011).
19. S. Sahreen, M.R. Khan and R.A. Khan, *BMC Complem. Alternat. Med.*, **11**, 48 (2011).
20. J. Reisch, R.A. Hussain and B. Krebs, *Montash. Chem*., **121**, 941 (1990).
21. S.K. Rai, G.R. Mallavarapu, S.P.-Rai, S. Srivastava, D. Singh, R. Mishra and S. Kumar, *Flav. Fragr. J.*, **21**, 304 (2006).
22. G.R. Mallavarupa, R.K. Mishra, S. Chaudhary, R. Pandey, S. Gupta, S. Kumar, V.K. Kaul and V. Pathania, *J. Essent. Oil Res*., **21**, 385 (2009).
23. B. Nickavar and N. Yousefian, *Iranian J. Pharm. Res.*, **8**, 53 (2009).
24. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk Susceptibility Tests: Fifteenth Informational Supplement M 100-S15. CLSI, Wayne, PA, USA, (2005).
25. D. Ahmed, M.A. Chaudhary, A. Raza, A. Waheed, S.R. Khan and M. Ikram, *Asian J. Chem.*, **24**, 68 (2012).
26. M.A. Chaudhary, D. Ahmed and S.R. Khan, *Ethnobotanical Leaflets,* **13**, 1088 (2009).
27. W. Brand-Williams, M.E. Cuvelier and C. Berset, *Lebens. Wissen. Technol*., **28**, 25 (1995).
28. D. Ahmed, M.A. Arshad, M.N. Asghar and M. Ikram, *Asian J. Chem.*, **22**, 4524 (2010).
29. M. Umamaheswari and T.K. Chatterjee, *Afr. J. Tradit. Complement. Alternat. Med.*, **5**, 61 (2008).
30. P. Bernfeld, *Enzymology*, **1**, 149 (1955).
31. Y. Matsumoto, A. Ikemoto and S. Tawara, *J. Infect. Chemother.*, **5**, 176 (2007).
32. H. Baig, D. Ahmed, S. Zara, M.I. Aujla and M.N. Asghar, *Oriental J. Chem.*, **27**, 1509 (2011).