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Nutritional and Antimicrobial Studies on Leaves and Fruit of *Carissa opaca* Stapf ex Haines

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Carissa opaca Stapf ex Haines is wild plant having a number of ethnomedicinal applications. In the present study, the leaves and fruits of the plant were subjected to antimicrobial and nutritional investigations. Antimicrobial activity of the ethanolic extracts of the leaves and fruits of the plant, determined against 34 strains of gram-positive and gram-negative bacteria, exhibits a considerable broad spectrum efficacy. Analysis of mineral elements present in fruits, seeds and leaves shows that the plant has good quantities of potassium, magnesium, iron, zinc, copper and chromium. The nutritional value of the fruits was 333.84 cal/100 g and they are a good source of fiber, lipids, protein and carbohydrates.

Key Words: *Carissa opaca*, Antimicrobial activity, Trace elements, Nutritional contents.

INTRODUCTION

Carissa opaca Stapf ex Haines belongs to family Apocynaceae. The genus Carissa comprises about 20 species two of which are found in Pakistan, namely, *C. carandas* and *C. opaca*. The latter is distributed in many mountainous parts of Indian subcontinent[1-3], from Punjab to Himalayas in Pakistan and India and Burma and Sri Lanka[4]. It is an evergreen, thorny shrub with spines arising between the petioles, leaves are glabrous, opposite, elliptic, ovate or rounded and about 1.0-3.5 cm long and the berries are somewhat ellipsoid or subglobose, 6-8 mm long, dark purple when ripe having milky juice[5]. The ripe fruits having a sweet-sour taste are eaten and are also used to make pickles and twigs are browsed by sheep and goats[6]. In ethnomedicine, *Carissa opaca* is used against a number of diseases and conditions. It is used to cure fever and eye disorders and the fruit of the plant mixed with roots of *Mimosa pudica* is taken as aphrodisiac[7,8]. It is used to cure jaundice and hepatitis. For this purpose fresh leaves of the plant and roots of *Segeretia brandrethiana* are boiled in water and a cup of the decoction is taken orally twice a day for 2-3 weeks[9]. A paste of the plant root is used by local people for healing small cuts and wounds. The plant is commonly used as a medicine to kill worm infesting cattle wounds, as fly repellent, as stimulant and to cure asthma and its leaves are also used for tanning[1,3,10-12]. The plant is known to have cardiotonic action while roots can be used as purgative. The plant is also used for the treatment of horn injuries and maggot wounds in animals and the root paste of the plant is applied locally[13]. In order to rationalize the ethnomedicinal applications of the plant we carried out nutritional and antimicrobial studies on fruit, seeds and leaves of *Carissa opaca*.

EXPERIMENTAL

Leaves and fruits of *Carissa opaca* Stapf ex Haines were collected from the hilly area near Abbottabad, Pakistan, in May 2009. The botanical identification of the plant was confirmed by the taxonomist of Hazara University, Professor Dr Syed Muqarrab Shah. A sample of the plant was kept at the Department of Chemistry, Forman Christian College, Lahore.

Extract preparation: The leaves and fruits were dried under shade for 2 weeks. For antimicrobial study the dried, powdered leaves (20 g) and fruits (15 g) were extracted with 95 % ethanol at room temperature (100 mL × 10 days × 3). The extracts were filtered and the filtrates were combined and the solvent was evaporated under reduced pressure using rotary evaporator to get dried ethanolic extracts of leaves and fruits. 3 g of each extract was then dissolved in 1,2-propanediol to prepare stock solutions (100 mL each).

Antimicrobial study

Microorganisms: In this study, 34 strains of various microorganisms were used, which included 6 ATCC reference

strains and 28 clinical isolates. Reference strains were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Acinetobacter baumannii* (ATCC 29212) and *Shigella sonnei* (ATCC). The clinical isolates included methicillin-resistant *Staphylococcus aureus* (MRSA, five strains), multidrug-resistant (MDR) *Salmonella typhi* (four strains), extended-spectrum β-lactamase (ESBL) *Escherichia coli* (three strains), *Staphylococcus aureus* (two strains), *Escherichia coli* (two strains) and one strain each of *Listeria spp., Micrococcus spp., Shigella sonnei, Citrobacter freundii, Escherichia hermannii, Vibrio cholerae, Salmonella typhi, Providencia rettgeri, Citrobacter koseri, Klebsiella pneumoniae, Serratia marcescens* and methicillin-resistant *Staphylococcus epidermidis* (MRSE). The reference strains and clinical isolates were provided by the Department of Microbiology, University of Health Sciences (UHS), Lahore, Pakistan and were identified by standard morphological, cultural and biochemical profile (API-20E, bioMerieux, France)[14]. The isolates were preserved in microbank tubes containing beads (Pro-Lab Diagnostics, UK) and 16 % (v/v) glycerol in brain heart infusion (Oxoid Ltd., UK) and were stored at -70 °C[15].

Agar well diffusion assay: Antimicrobial susceptibility of the leaves and fruit extracts of *Carissa opaca* was evaluated by agar well diffusion assay according to method recommended by Clinical and Laboratory Standards Institute (CLSI)[16] with slight modification. In each experiment, the bacterium under test was spread on the Mueller-Hinton Agar (MHA) medium (Merck, Germany) in a Petri dish with the help of a wire loop (10 µL). The Petri dish was incubated at 37 °C for 24 h to obtain uniform well-isolated colonies of the bacterium. To prepare a bacterial suspension, 4-5 morphologically identical colonies of the bacterium were transferred into a test tube containing autoclaved normal saline (NS) solution (5 mL) with the help of the wire loop. Then the content of the test tube was shaken and its turbidity was matched with 0.5 McFarland solution used as a standard.

Inoculation of bacteria: With the help of a sterile cotton swab, the bacterial suspension was then carefully transferred into Petri dishes (90 mm diameter) containing a thoroughly mixed and autoclaved Mueller-Hinton Agar medium (20 mL each). The plates were allowed to dry for a few minutes at room temperature. With the help of a sterile cork borer (9 mm diameter) equally spaced four wells were made in each Mueller-Hinton Agar plate, two for leaves and fruit extracts and one each for positive and negative controls. The material from the wells was removed by using a sterile syringe needle. The wells were then labeled and with the help of micropipettes, 140 µL of each extract and controls were transferred into their corresponding wells. Phenol (6 %) and 1,2-propanediol (50 %) were used as positive and negative control, respectively. Then the plates were incubated at 37 °C for 24 h, after which zones of inhibition (ZI) were measured with the help of digital Vernier calipers (Sylvac, Fowler, Ultra-Call11). Each test was repeated three times and the average calculated.

Agar dilution assay to determine MICs: The minimum inhibitory concentration (MIC) is the lowest concentration of an antibacterial agent that inhibits the growth of a bacterium. This provides a quantitative measure of antimicrobial activity of a substance. For this purpose, solutions of different concentrations of the substance under study are prepared and tested for antimicrobial activity. Agar dilution assay as described by CLSI[16] was used to determine MIC values for the leaves and fruit extracts of *C. opaca*. Two-fold dilutions of leaves and fruit extracts were prepared to obtain final concentrations in Mueller-Hinton Agar medium (50 °C) as 1.5, 3.0, 4.5 and 6.0 mg/mL in 20 mL volume. One mL of leaves or fruit extract (30 mg) was mixed with 19 mL of Mueller-Hinton Agar and 2 mL of the extract was mixed with 18 mL of Mueller-Hinton Agar and so on. Each dilution, in a Petri plate (90 mm diameter), was allowed to dry at 45 °C for *ca.* 10-15 min. These plates were then inoculated using a multipoint inoculator (Mast Diagnostic, UK; having 35 points). For this purpose, 4-5 well isolated colonies from overnight blood agar were emulsified in 5 mL of sterile distilled water and the turbidity was adjusted to 0.5 McFarland's standard. The inoculator immersed in a bacterial culture (3 µL) transferred the organism onto the agar plates. Three control plates were also set up in parallel, one of Mueller-Hinton Agar without extract inoculated with all strains to confirm the viability of the cultures; second containing medium only and the third having both the medium and extract to test the sterility of both the medium and the extract. The plates kept in the incubator for *ca.* 24 h were observed and in each case the MIC was recorded as the lowest concentration of the extracts at which visible bacterial growth was completely inhibited. The experiment was performed in triplicate to ensure the reproducibility.

Determination of mineral elements: The mineral elements were determined in the leaves, fruit pulp and seeds of *C. opaca* by the method described earlier[17]. The atomic absorption spectrophotometer used for mineral elements analysis was Varian Model AA240 (AAS). In each case, a weighed sample (5.0 g) was heated on flame to remove moisture and volatile matter. Then it was heated in a furnace at 600 °C for *ca.* 4 h which converted the sample into ash. The ash was dissolved in conc. HNO_3 (12 mL) and the total volume was made 100 mL by adding twice distilled water. The content was then filtered to get a clear solution which was used for the analysis. The experiment was done in triplicate and the results were averaged.

Estimation of nutritional values: The nutritional values (moisture, ash, crude fat, crude protein, carbohydrates and crude fiber) of the whole fruit of *C. opaca* were estimated using standard methods[18]. The moisture content was determined by oven drying of the weighed sample to a constant weigh at 105 ºC, for *ca.* 2 h. The crude fat was determined by Soxhlet extraction with hexane (60 ºC) for 6 h. Crude protein was estimated by Kjeldahl method. Carbohydrates were determined by difference [% Carbohydrates = 100 – (% Ash + % Moisture + % Fat + % Protein)].

1.5 g of oven dried sample was burnt in a muffle furnace at 550 ºC temperature for 10 h until white ash was obtained,

which was used to calculate the percentage of ash. To determined crude fiber, the weighed sample was refluxed with 1.25 % H_2SO_4 for 0.5 h. It was then filtered and the residue was washed with hot water and then refluxed with 1.25 % NaOH for 0.5 h. The content was filtered with an ashless filter paper of known weight and rinsed with distilled water. The oven dried residue was weighed and then charred in a muffle furnace to get white ash, which was weighed to calculate fiber. Nutritive value was determined by the formula:

Nutritive value = 4 × % Protein + 9 × % Fat + 4 × % Carbohydrate.

RESULTS AND DISCUSSION

Antimicrobial studies: The results of antimicrobial studies on leaves and fruit extracts of *C. opaca* Stapf ex Haines against various strains of reference and clinically isolated bacteria are presented in Table-1 (zones of Iinhibition in agar well diffusion assay) and Table-2 (minimum inhibitory concentrations). The results of antimicrobial studies show that the ethanolic extract of *C. opaca* possesses a broad spectrum of activity against both the gram-positive and gram-negative strains of reference and clinical isolates. As the Table-2 shows ethanolic leaves extract of *C. opaca*, in general, exhibited better antimicrobial activity than fruit extract. Table-1 reveals that the clinical isolate of *S. typhi* MDR strain 3 has proved to be most susceptible to all the extracts. The extracts also showed higher toxicity against the other *S. typhi* MDR isolates as well as *S. aureus*. The fruit extract showed the highest antibacterial activity against strain 3 of clinical isolate of MDR *S. typhi* with zone of inhibition of 28 mm. As the MIC values indicate (Table-2), leaves of *C. opaca* have excellent efficacy against a number of deadly pathogens including *A. baumannii* (ATCC 29213), all the strains of MRSA, MRSE, *S. aureus*, *Micrococcus spp., S. marcescens, V. cholerae* and *S. typhi*. Since the extracts are as much effective against gram-positive *S. aureus* as gram-negative *S. typhi*, they are apparently unable to distinguish between gram-positive and gram-negative bacteria.

TABLE-1
ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT* OF LEAVES AND FRUIT PULP OF *Carissa opaca* (DIAMETER OF INHIBITION ZONES IN mm)**

Test microorganism	Leaves		Fruits	
	Extract	6 % Phenol	Extract	6 % Phenol
Pseudomonas aeruginosa (ATCC 27853)	18.5	30.5	17.5	30.5
Staphylococcus aureus (ATCC 25923)	23.1	30.0	19.3	30.0
Escherichia coli (ATCC 25922)	15.1	23.8	15.2	23.8
Acinetobacter baumannii (ATCC 29213)	17.2	32.0	13.4	32.0
Enterococcus faecalis (ATCC 29212)	16.5	21.0	19.0	21.0
MDR *Salmonella typh*i strain 1	21.5	32.0	21.5	32.0
MDR *Salmonella typhi* strain 2	23.1	30.0	17.5	30.0
MDR *Salmonella typhi* strain 3	24.5	30.0	28.0	30.0
MDR *Salmonella typhi strain* 4	20.2	30.0	16.5	30.0
MRSA strain 1	18.0	26.0	15.0	26.0
MRSA strain 2	18.5	26.0	14.5	26.0
MRSA strain 3	18.5	26.0	14.5	26.0
MRSA strain 4	17.5	26.0	17.2	26.0

*Concentration of leaves and fruit was 3 mg/mL.
**The values are the mean of three experiments.

TABLE-2
MINIMUM INHIBITORY CONCENTRATIONS (MICs) OF ETHANOLIC EXTRACTS OF LEAVES, FRUIT AND SEEDS OF *Carissa opaca* AGAINST DIFFERENT PATHOGENS

Test microorganism	MICs* (mg/mL)	
	Leaves	Fruits
Acinetobacter baumannii ATCC 29213	1.5	3.0
Shigella sonnei ATCC	3.0	3.0
Enterococcus faecalis ATCC29212	3.0	3.0
Pseudomonas aeruginosa ATCC 27853	3.0	3.0
Escherichia coli strain 1	3.0	3.0
Escherichia coli strain 2	3.0	3.0
Escherichia coli- ESBL strain 1	3.0	3.0
Escherichia coli- ESBL strain 2	3.0	3.0
Escherichia coli- ESBL strain 3	3.0	3.0
Salmonella typhi	1.5	3.0
MDR-*Salmonella typhi* strain 1	3.0	3.0
MDR-*Salmonella typhi* strain 2	1.5	3.0
MDR-*Salmonella typhi* strain 3	3.0	3.0
MDR-*Salmonella typhi* strain 4	3.0	3.0
MRSA strain 1	1.5	3.0
MRSA strain 2	1.5	3.0
MRSA strain 3	1.5	3.0
MRSA strain 4	1.5	3.0
MRSA strain 5	1.5	3.0
MRSE	1.5	3.0
Staphylococcus aureus	1.5	3.0
Micrococcus spp.	1.5	1.5
Shigella sonnei	3.0	3.0
Citrobacter freundi	3.0	3.0
Serratia marcescens	1.5	1.5
Klebsiella pneumoniae	3.0	3.0
Listeria spp.	3.0	3.0
Escherichia hermannii	3.0	3.0
Providencia rettgeri	3.0	3.0
Vibrio cholerae	1.5	3.0
Citrobacter koseri	3.0	3.0

*MIC values are the mean of triplicate determination.

The plant thus has wide range of antimicrobial properties. It should particularly be effective in typhoid which is still a major health problem in the world[19,20]. The fruit is also effective against *Enterococcus faecalis* (ATCC 29212) which causes skin and soft tissue infections[21]. *Staphylococcus aureus* (MRSA) is the causative agent of several human diseases including the septic arthritis, endocarditis, staphylococcal scalded skin syndrome[22] and it also acts as an ocular pathogen[23]. The antimicrobial activity of the extracts may be attributed, in part, to the presence of phenolic compounds[24] whose presence in this plant has been established in a recent study[25].

Nutritional studies: The nutritional parameters (moisture, ash, fiber, protein, fat and carbohydrates) of the fruits of *C. opaca* were determined and the results were presented in Table-3. As is obvious from the Table-3, *C. opaca* fruit is a good source of various nutrients. It contains considerable quantities of vegetable fats and proteins and good quantity of carbohydrates and fiber. Consumption of 100 g of the fruit can give an individual 333.84 calories of energy. All these values along with mineral elements and antimicrobial potential make this wild fruit nutraceutically important.

Mineral elements studies: Mineral elements (Na, K, Mg, Fe, Cu, Zn, Cr, Ni) in the leaves, fruit pulp and seeds of *C. opaca* were determined with atomic absorption spectropho-

TABLE-3
NUTRITIONAL VALUES OF THE FRUIT OF *Carissa opaca*

Nutritional parameter	Amount
Moisture	15.00 %
Ash	4.78 %
Fiber	13.55 %
Crude Fat	13.40 %
Crude Protein	6.31 %
Crude Carbohydrates	47.00 %
Nutritional value	333.84

tometer and the results are shown in Table-4. As the table reveals, the leaves, fruit pulp and seeds of *C. opaca* contain comparable amounts of Na, but the amount of K is almost two times higher in fruit pulp and seeds than that in leaves. Moreover, K is about four times higher than Na in fruit pulp and seeds. Thus the consumption of the whole fruit of the plant would maintain the balance in favour of K, which is important for normal blood pressure[26]. While Mn is contrastingly higher in leaves than fruit pulp and seeds, the seeds are richer in Cr. Quite interestingly fruit pulp was found to contain no Cr. Thus, consumption of seeds can benefit patient of diabetes as Cr plays a role in stabilization of blood sugar level[27]. The fruit and seeds also contain significant amount of Cu (more than 19 ppm), which is a component of various enzymes and plays role in the synthesis of collagen, regulation of normal cardiovascular and immune functions[28]. The samples contain good quantities of Mg, Fe, Zn and Ni. All these elements play important role in human body. Magnesium is a component of bones and many enzymes and plays important role in the regulation of blood sugar levels and blood pressure and is necessary for the transmission of nerve impulses, which affects contraction and relaxation of muscles[29-38]. Iron is a component of many enzymes and is an essential part of hemoglobin (blood protein) and myoglobin (muscle protein)[39,40]. Zinc is a component of about 70 enzymes and plays a variety of roles. It promotes proper growth along with sexual maturity[41]. The role of zinc in eye health is important. It has been shown to play an integral role in maintaining normal ocular function[42]. It also possesses antioxidant properties[43]. Manganese is another essential trace element which is required by human body in low quantity[38,40]. No sample was found to contain lead and cobalt.

TABLE-4
AMOUNT OF MINERAL ELEMENTS IN LEAVES, FRUIT PULP AND SEEDS OF *Carissa opaca* (ppm)

Element	Leaves	Fruit pulp	Seeds
Na	923.49	1086.99	991.36
K	2253.5	4176.94	4242.79
Mg	762.75	718.62	726.46
Fe	353.95	250.68	213.03
Cd	1.00	0.58	1.04
Ni	8.25	4.51	4.10
Cu	9.81	19.94	19.60
Zn	32.00	26.00	52.00
Mn	99.54	29.03	37.44
Cr	3.00	Nil	53.00
Pb	Nil	Nil	Nil
Co	Nil	Nil	Nil

Conclusion

The *in vitro* antimicrobial studies present *C. opaca* to have considerable efficacy against various pathogenic bacteria. The study provides a scientific basis for the use of the plant as folk medicine. The fruit of the plant is a good source of essential nutrients including minerals, carbohydrates, proteins and lipids. However, more advanced pharmacological and clinical studies would be required to investigate *in vivo* mechanism of nutraceutical effects of this important wild plant.

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