SUPPLEMENTAL MATERIAL

Isolation and identification of bioactive compounds from chloroform fraction of methanolic extract of *Carissa opaca* roots

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Abstract

Carissa opaca is a shrub known for its variety of medicinal applications. The present study reports isolation and identification of four chemical compounds from its roots for the first time. The methanolic extract of the roots was fractionated into various solvents with increasing polarity. Chloroform fraction was subjected to column and thin layer chromatography to ultimately yield 2*H*-cyclopropanaphthalene-2-one, 7-hydroxy-6-methoxy-2*H*-1-benzopyran-2-one, 3-(4-methoxyphenyl)-2,6-dimethylbenzofuran, and 5(1*H*)-azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8*S*-cis). They were identified by GC-MS analysis. The compounds exhibited considerable antimicrobial activities against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus niger* with zones of inhibition ranging from 10-13 mm as compared to the standard drug Amoxicillin with zones of inhibition 13-17 mm under the similar conditions. In conclusion, the roots of *C. opaca* can provide new leads for future antimicrobial drugs.

Key Words: *Carissa opaca*, roots, compounds, antibacterial, antifungal

Experimental

Plant Collection

The roots of *Carissa opaca* were collected from the hills near Abbottabad, Pakistan, in March 2013. The identification of the plant was confirmed by the taxonomist Professor Ajaib Khan of GC University, Lahore, Pakistan (voucher specimen: GC-Herb Bot 2271).

Extraction and Preparation of samples

The roots were washed with distilled water to remove soil particles, and dried under shade for two days. They were crushed and ground to get a fine powder. Extraction and fractionation was carried out by a method commonly employed [Choi et al. 2009; Bibi et al. 2010; Shahzadi et al. 2011]. The powder (7 kg) was then macerated in pure methanol in a glass bottle for 15 days, with frequent shaking. The extraction was repeated for two more times in order to have maximum extraction. The solvent was evaporated on rotary evaporator under reduced pressure to obtain dried methanolic extract (600 g) of the roots. A portion of the methanolic extract (500 g) was

suspended in distilled water, and placed in a separating funnel. It was fractionated successively into solvents with increasing polarity. Extraction into each solvent was repeated three times. As a result, hexane (40.1 g), chloroform (20.9 g), ethyl acetate (30.55 g), *n*-butanolic (90.6 g) and residual aqueous (315 g) fractions were obtained upon evaporation of the solvents.

Chemicals

The organic solvents used in the present work were of HPLC grade. DMSO (dimethyl sulfoxide) was purchased from Fisher Scientific (Loughborough, UK), Mueller-Hinton agar, potato dextrose, and TLC plates (silica gel) from Merck (Kenilworth, USA). Standard antibiotic Amoxicillin was obtained from Pharmagen Pharmaceuticals, Lahore, Pakistan. All other chemicals used were of analytical grade.

GC-MS Equipment

GC-MS was Agilent GC7890A/MS5975. The gas chromatography equipped with a HP-5 MS. The carrier gas was helium at flow 0.8 mL/min. The temperature of the column was 60 °C for zero min 5 °C/min to 70 °C and 10 °C/min to 310 °C for 4 min.

Microorganisms

Microorganisms used for the study included three bacteria *Bacillus subtilis* ATCC6633 (Gram +), *Escherichia coli* ATCC8739 (Gram -), *Pseudomonas aeruginosa* ATCC9027 (Gram -), and two fungi *Candida albicans* ATCC10231 and *Aspergillus niger* ATCC16404. All the standard stains were purchased from KWIK-STICK.

Isolation of phytochemicals

Chloroform fraction of methanolic extract of the roots of *Carissa opaca*, which exhibited considerable bioactivities, was subjected to chromatographic techniques to isolate and identify its chemical constituents.

Column chromatography and TLC

Chloroform fraction was chromatographed on a glass column (height 60 cm, diameter 6.35 cm) using silica gel (mesh 100-200) as adsorbent using the wet loading method. Column was washed with distilled water and rinsed with hexane to get it completely dried. A slurry of the silica gel, prepared in hexane in a usual method, was carefully poured into the column, which had a glass wool stopper at the lower end. A paste of the chloroform fraction was prepared in hexane and poured into the column to form a layer at the top of silica gel bed. It was then covered with a layer of silica gel slurry. Elution was carried out with a mixture of hexane-ethyl acetate with gradually increasing polarity (Table 1) [Omoyeni 2014]. The solvent system of each ratio consisted of 200 mL (Table S1). Based on the similarity of these fractions in their TLC behavior, similar fractions were combined. Since the sub-fractions FC, F10 and F11 were of considerable quantities, they were selected for phytochemical investigation. Each of them was subjected to thin layer chromatography. **COC1** was isolated from F11, which appeared as major spot on TLC at R_f value of 0.77. **COC2** was isolated from F10 as yellow crystals, which were washed with ethyl acetate and re-crystallized from methanol. **COC3** and **COC4** were isolated from the sub-fraction FC. On TLC, two major bands were found at R_f 0.27 and 0.35. The former was

designated as **COC3** and the later as **COC4**. The solvent systems used for column chromatography and TLC are shown in Table S1.

Identification of compounds by GC-MS analysis

The pure compounds were identified by GC-MS. The GC chromatogram of each compound showed single peak demonstrating the purity of the isolated compounds (Tables S2 and S3). The mass spectra matched suggesting the identification of the compounds.

Study of antimicrobial activity

Antimicrobial activities of the isolated compounds were determined by the common agar well diffusion method as reported earlier [Perez et al. 1990; Dar et al. 2014]. Briefly, Mueller-Hinton agar, prepared in distilled water, autoclaved at 121 °C for 1 h. Distilled water was added to a lyophilized culture, which was incubated for 24 h at 32.5 °C. Saline solution was added into the slant containing the culture. Its absorbance was standardized with 0.5% McFarland standard solution at 550 nm. Then, 2 mL of this diluted suspension was added into 100 mL liquid agar. Growth on the surface of slant was washed with 20 mL 0.9% saline solution and the mixture was heated at 70 °C for 30 min. The Mueller-Hinton agar (21 mL) was poured into a 100-mm Petri plate and allowed to solidify. As soon as it solidified, 4 mL seed agar was poured onto it to set a thin layer. The Petri plate was placed in a refrigerator for cooling for 1 h. Wells were dug into it and labelled. Solutions of the isolated compounds were prepared in DMSO (1 mg/mL), and filled in the wells. The plates were then incubated for 24 h, after which, they were observed for antimicrobial activity of the samples, and zones of inhibition were measured. For antifungal activity, growing medium used was consisted of potato dextrose agar, and temperature was maintained at 30.5 °C. The incubation of 7 days was required in case of *A. niger* for full propagation of spores. Zones of inhibition are exhibited in Table S4.

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Tables

Table S1: Sub-fractions obtained from column chromatography (CC) at solvent systems with increasing polarity and mobile phase for TLC used to isolate compounds.

S#	Sub-fractions	CC solvent systems	TLC solvent systems	Compounds isolated
1	FA	100% hexane, and hexane: ethyl acetate (9:1)	-	-
2	FB	hexane: ethyl acetate (8:2) and (7:3)	-	-
3	FC	hexane: ethyl acetate (6:4), (5:5) and (4:6)	hexane: ethyl acetate (4:6)	COC3, COC4
4	F8	hexane: ethyl acetate (3:7)	-	-
5	F9	hexane: ethyl acetate (2:8)	-	-
6	F10	hexane: ethyl acetate (1:9)	Not needed	COC2
7	F11	100% ethyl acetate	hexane : ethyl acetate (4:6)	COC1

Table S2: Chemical compounds isolated from *Carissa opaca* and identified by GC-MS, their retention times (RT), and bioactivities.

Compound Code	RT (min)	Name	MF	MW	Bioactivity
COC1	21.732	2 <i>H</i> -Cyclopropanaphthalene-2-one	C ₁₅ H ₂₂ O	218	Antifungal, antibacterial
COC2	19.274	7- Hydroxy- 6- methoxy-2 <i>H</i> -1-benzopyran-2-one	C ₁₀ H ₈ O ₄	192	Antifungal, antibacterial
COC3	20.336	3-(4-methoxyphenyl)-2, 6-dimethylbenzofuran	C ₁₇ H ₁₆ O ₂	252	Antifungal, antibacterial
COC4	21.712	(5(1 <i>H</i>)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-,(8 <i>S</i> -cis)	C ₁₅ H ₂₂ O	218	Antifungal, antibacterial

MW molecular weight, MF molecular formula

Table S3: Isolated compounds by column chromatography from chloroform fraction of *Carissa opaca* roots extract.

Table S4: Antimicrobial activities of compounds isolated from chloroform fraction of *Carissa opaca* roots extract in terms of zones of inhibition (mm).

Compound	BS	EC	PA	CA	AN
COC1 (2H-Cyclopropanaphthalene-2-one)	11	13	10	11	12
COC2 (7-Hydroxy- 6- methoxy-2H-1-benzopyran-2-one)	11	12	12	nd	11
COC3 (3-(4-Methoxyphenyl)-2,6-dimethylbenzofuran)	11	11	nd	nd	12
COC4 (5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)	11	13	12	nd	nd
Amoxicillin (Standard drug)	15	17	15	14	13

BS *Bacillus subtilis*, EC *Escherichia coli*, PA *Pseudomonas aeruginosa*, CA *Candida albicans*, AN *Aspergillus niger*; nd not detected.